PHYSIOLOGICAL INDICATORS OF TICK-INDUCED STRESS IN GRAZING ANIMALS: DETECTION BY NON-INVASIVE BIO-FORENSIC TECHNIQUES

A Dissertation

by

DOUGLAS RAY TOLLESON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Rangeland Ecology and Management

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Approved by:

Co-Chairs of Committee,	William E. Pinchak
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December 2007

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ABSTRACT

Physiological Indicators of Tick-induced Stress in Grazing Animals:

Detection by Non-invasive Bio-forensic Techniques.

(December 2007)

Douglas Ray Tolleson, B.S., Texas A&M University; M.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. William E. Pinchak
Dr. Fred E. Smeins

Three studies utilizing a single group of growing beef steers were conducted to ascertain the effects of tick stress on cattle and to evaluate the use of bio-forensic techniques of detection. Steers (n = 28, 194 ± 3.0 kg) were randomly assigned to one of four treatments in a 2 x 2 factorial arrangement: moderate (14.0 ± 1.0% CP, 60 ± 1.5% TDN) versus low (7.0 ± 1.0% CP, 58 ± 1.5% TDN) plane of nutrition, and control (no tick) versus tick treatment (300 pair of adult (*Amblyomma americanum*) per treated animal). Steers were individually fed experimental diets *ad libitum* for 35 days prior to and 21 days following the start of tick infestation (day 0), with peak tick feeding occurring 10 to 14 days post tick infestation. In study 1, blood was sampled on day -7, 0, 7, 8, 9, 10, 11, 13, 17 and 21, and plasma analyzed for metabolic and endocrine indicators. Within the low plane of nutrition, IGF-1 (ng/ml) was greater in control ($P < 0.05$) than in the tick treated (139.57 ± 9.3 vs 111.4 ± 9.3) group. Within the moderate plane of nutrition, tick treated cattle had higher ($P < 0.05$) plasma cortisol than non-treated. In study 2, fecal samples were analyzed for metabolic, endocrine and immunologic indicators. Fecal cortisol was the only constituent measured that was

affected by treatment and not by plane of nutrition. The highest average daily fecal cortisol observed was for day 13, during peak tick feeding and after six days of repeated blood sampling. In study 3, near infrared spectra were obtained in the 1100-2498 nm range. Spectra were assembled into groups by plane of nutrition, treatment, and by plane of nutrition by treatment. Periods of 7 ± 1 days correspond to significant delineations in the tick feeding cycle. There were differences in pre-infestation versus infestation fecal spectra within the tick treated groups in both the moderate and low planes of nutrition. These differences can not be wholly attributed to tick treatment, but may have also been affected by blood sampling stress.

DEDICATION

This work is dedicated to two well respected men; if I accomplish half of what they did in life, I will consider myself a success:

Roy Lee Tolleson

Father and Friend

Born: September 1, 1941

Died: May 1, 2006

You taught me to work and to be a man. I will never forget when I was kid and you said to me *"Boy, don't ever be afraid to learn something… you never know when you might need it."*

Jerry Wayne Stuth

Professor and Friend

Born: November 4, 1947

Died: April 24, 2006

You helped me develop into a scientist and to see the world… literally. Just one more time I wish you could storm into my office and say *"Doug, how hard would it be to…?"*

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you volunteered to be on the committee as soon as you knew I needed one, and always treated me like a colleague.

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Mattie, if you could read, I would thank you too. How about I just pat you on the head and let you ride in the truck?

Lana, Ross, Marshall… Sure, this is an accomplishment and milestone for me, but don't ever forget, I did this mostly for you.

I am not that much of a Willie Nelson fan anymore but I think this song says it pretty well:

"It's been rough and rocky travelin', But I'm finally standin' upright on the ground.

After takin' several readings, I'm surprised to find my mind`s still fairly sound."

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CHAPTER I

INTRODUCTION: THE PHYSIOLOGY OF TICK-INDUCED STRESS IN GRAZING ANIMALS

Introduction

Stress can be defined as a threat to homeostasis, some event which the body perceives as harmful and thus requires defensive action. The concept of stress in biological terms was first proposed by Selye (1936). His observation that rats receiving frequent injections developed (among other symptoms) enlarged adrenals regardless of what the injections contained, led others to determine that the hypothalamus, the anterior pituitary, and the adrenals function to produce hormones responsible for the physiological actions now known as the "stress response". In general this response involves those processes which prepare the body for "fight or flight". Included among these processes are: tachycardia, vasoconstriction in the skin, vasodilation in skeletal muscle, and cessation of "non-essential" processes such as digestion or immune function in the short-term.

The endocrinology of stress involves catecholamines released from the adrenal medulla as a result of sympathetic nervous system stimulation and, a feedback driven cascade of

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This thesis follows the style of the Journal of Animal Science.

hormones produced by an interrelated group of tissues known as the hypothalamic-pituitary-adrenal axis (HPA). Briefly, upon perception of a stressor, the hypothalamus secretes corticotrophin releasing factor (CRF) which acts upon the anterior pituitary to cause adrenocorticotropic hormone (ACTH) production. The adrenal cortex subsequently releases corticosteroids in response to ACTH. Some of the physiologic affects of acute stress were previously alluded to; heightened awareness, increased heart rate and muscle tone, all positive developments if the individual is being called upon to defend itself or escape. Chronic stress, however, can be detrimental to homeostasis. Classic work by Sopolsky (2003) with baboons has illustrated the long-term effects of stress on overall health and well-being. Immunity is, of course, a significant player in this process.

The Immune System

One of the most exciting and rapidly expanding fields in the life sciences is immunology. Though driven primarily by interest in combating disease, research into the immune system has also helped expose the intricate connections among various systems in the body. Disciplines such as psychoneuroimmunology, for instance, deal with the actions of such neuroendocrine tissues as sympathetic neurons and HPA on metabolism and the immune system (Ader et al., 1991). In addition to indirectly affecting immune function by potentially shunting resources away from immune tissues

during a stressful situation, direct effects of glucocorticoids on various immuno-active cells have been demonstrated (Sheridan et al., 1994). Mononuclear cells contain receptors for glucocorticoids (Werb et al., 1978). Glucocorticoids inhibit major histocompatibility II expression (Zwilling et al., 1992) and tumor necrosis factor production (Beutler et al., 1986) in macrophages. Cortisol (Brown-Borg et al., 1993) reduced lymphocyte production in swine. Hormones of non-adrenal origin have direct effects on immune tissues as well. Growth hormone and insulin-like growth factor 1, for instance, in addition to their better-known metabolic functions, also expedite maturation of lymphocytes in bone marrow (Merchav et al., 1988) and thymus (Berschorner et al., 1991). The communication between the immune system and other physiological systems is bi-directional. Cytokines, the chemical messengers produced by immune cells, influence many metabolic functions; for example protein synthesis and muscle maintenance. Interleukin-1, Interleukin-6, and tumor necrosis factor-α are among the cytokines considered to be "pro-inflammatory". Insulin-like growth factor-1, both circulatory and intramuscular, is reduced by elevated concentrations of these cytokines (Ferrucci and Guralnik, 2003). Thyroid hormones are important regulators of basal metabolic rate. Pro-inflammatory cytokines diminish the response of thyroid-stimulating hormone to thyrotropin-releasing hormone (Wolf et al., 1994), thus affecting peripheral thyroid hormone levels and ultimately basal metabolism.

Stress and the Immune System

Venkatraman and Pendergast (2002, pp 323-324) in a review concerning the effects of stress on the immune system state:

> …stress leads to a proportional increase in stress hormone levels and concomitant changes in several aspects of immunity, including the following: high cortisol; neutrophilia; lymphopenia; decreases in granulocyte oxidative burst, nasal mucociliary clearance, natural killer cell activity, lymphocyte proliferation, the delayed-type sensitivity response, the production of cytokines in response to mitogens, and nasal and salivary immunoglobulin A levels; blunted major histocompatibility complex II expression in macrophages; and increases in blood granulocyte and monocyte phagocytosis, and pro- and anti-inflammatory cytokines. In addition to providing fuel for exercise, glycolysis, glutaminlysis, fat oxidation and protein degradation participate in metabolism and synthesis of the immune components. Compromising, or overusing, any of these components may lead to immunosuppression.

Specifically, chronic stress suppressed IgG titer to keyhole limpet hemocyanin in hamsters (Jasnow et al., 2001). Matalka (2003) reviews evidence that acute stress induces the production of pro-inflammatory cytokines (i.e. promotes a T_h1 response), while chronic stress causes dysregulation in immune function by shifting the cytokine pattern to favor a T_h2 response. Chronic heat stress reduced white blood cell counts and antibody production in laying hens (Mashaly et al., 2004), while acute cold stress enhanced the innate immune response in growing chicks (Hangalapura et al., 2004). Beef steers grazing endophyte infected fescue have been reported to exhibit decreased phagocytic activity and expression of major histocompatibility complex II (Saker et al., 1998). However, Rice et al. (1997) found that antibody titer to Conconavilin A tended to be higher, and was higher ($P < 0.05$) against sheep red blood cells in cattle grazing infected fescue versus those consuming the non-infected variety. The effects of “stress”

on immunity is thus not a "one size fits all" phenomenon. Rook (1999) states that feedback from cytokines on the HPA axis helps the body control the immune response. Cortisol plays an important role in regulating immunity and inflammation, but as with many other vital physiological functions a delicate balance must be maintained; either too much or not enough can be harmful.

Gastrointestinal Tract

The gastrointestinal tract (GIT) is a hollow tube extending from oral cavity to anus. The most well known function of the GIT is digestion, absorption, and excretion of food residues. Basic architecture varies among species but in mammals generally the components are: mouth, esophagus, stomach, small intestine, cecum, large intestine, and associated structures such as the liver, pancreas, and gall bladder. The small intestine is the primary area for immune activity in the GIT; accordingly the discussion in this paper will focus there.

The small intestine is composed of three sections: the duodenum, jejunum, and ileum. It is the longest section of the GIT (~ 50 m bovine, 4 m canine, 6 m human) and the major site of nutrient absorption. The duodenum, jejunum and proximal ileum are where most digestion and absorption occurs (Pond et al., 1995). On cross-section the small intestine consists of longitudinal and circular outer muscular layers, followed by sub-mucosa and finally mucosa towards the lumen. The mucosa is the "front line" for both nutrient

absorption and immunity. The absorptive surface area of the small intestine is greatly expanded by: 1) folding of the sub-mucosal layer (*valvula conniventes*), 2) upon these folds are found villi, finger like outward projections 3) the villi are in turn lined with epithelial cells possessing a luminal cell surface covered by microvilli. The microvilli are also referred to as the brush border. The net result of the above convolutions and projections is a surface area of approximately 250 m^2 in the adult human (Guyton and Hall, 2000). Columnar absorptive cells, enterocytes, line the lumen of the small intestine. These are replaced every 3-5 days by new cells produced from simple tubular glands between the villi called crypts of Lieberkuhn. The second most numerous cells found here are goblet cells, important for their mucus secreting functions.

Digestion beyond that which occurs in upper sections of the GIT is carried out by specific enzymes on the cell surface of the enterocytes, in a layer of glycoproteins known as the glycocalyx. The net result is a population of molecules of a size and configuration appropriate for absorption. Absorption is often accomplished by nutrient dependent mechanisms coupled to NA/K transport, an energy consuming process which is responsible for much of the net energy of maintenance in animals. Undigested and unabsorbed feedstuffs are passed along the tract through the colon, and ultimately excreted from the anus along with resident microbes, various digestive secretions and sloughed epithelial cells (Church, 1979; Van Soest, 1983).

As important as the digestive functions of the GIT are to the animal, they are by no means the only important functions. The body is exposed to many potential antigens through ingestion of food and associated microbes. The gut epithelia is often described as being in a constant controlled state of inflammation. As such, the commensal microbial population exists in a delicate balance with the body's immune defenses.

Immune Function of the Gastrointestinal Tract

The GIT imposes three barriers to infection: 1) physical structure, 2) innate immune system, and 3) adaptive immune system. Each is interrelated and dependant upon the other two. The physical barrier (Nicoletti, 2000) consists of primarily the mucosa and associated layers. The epithelia, as previously mentioned, lines the lumen of the tract and comes into immediate contact with microbes and digesta. Tight junctions between cells here allow passage of water and ions but effectively block diffusion of macromolecules and microorganisms. The glycocalyx of columnar epithelial cells and mucus from goblet cells help shield the inner layers from microbial invasion. Paneth cells are found at the base of luminal crypts and have both secretory (IgA) and phagocytic (lysozyme) functions. Also found in the epithelia are membranous or microfold (M) cells (Kiyono and Fukuyama, 2004), which have specialized adaptations for transporting antigens. The lamina propria is the site of Peyer's patches (discrete clusters of lymphoid follicles), and in the sub-mucosa are numerous primary follicles as well as active germinal centers.

Paneth cells, M cells, and Peyer's patches are thus not only part of the physical barrier to infection, but also play an active part in the innate immune barrier.

Innate Immunity in the Gastrointestinal Tract

Innate immunity occurs in the gut associated lymphoid tissue. This somewhat amorphous collection of cells is designed to not only defend against intrusion by potentially harmful microbes, but perhaps equally significant, to not attack helpful organisms. Several cell types and mechanisms within cells exist to accomplish this unique balancing act. Nicoletti (2000) has recently reviewed the origin, structure, and function of M cells. These special cuboidal epithelial cells can be found adjacent to enterocytes but posses a distinct morphology. M cells exhibit considerably less microvilli than do other epithelial residents and the characteristic thick glycoclayx is noticeably absent. Such adaptations facilitate active phagocytosis of large particulate matter within their area. This feature obviously serves to eliminate dangerous bacteria by internalizing and presenting them to macrophages, but also exposes the body to exploitation by certain microbes which use this route to gain entry to underlying tissue (Kraehenbuhl and Neutra, 2000; Neutra et al., 1996). Another interesting feature which differentiates M cells from prototypical intestinal epithelial cells is the deep invagination of the basolateral membrane. Intraepithelial pockets are thus formed which create intimate contacts between the M cell and specific antigen presenting cells (APC) which

home to this area. Such modification of the basolateral area also results in shortened transit for endocytotic vesicles within the M cell.

In addition to being interspersed throughout the gut epithelia, M cells form areas of high concentration (>50%) immediately above the Peyer's patches. These areas are referred to as follicle-associated epithelium (Alpan et al., 2001). Kiyono and Fukuyama (2004) have presented a thorough review of Peyer's patch origin, structure, and function. Peyer's patches are dome-shaped structures within the lamina propria. This tissue is somewhat analogous to the deep cortex and follicles of a lymph node but with no afferent or efferent lymphatic vessels (Parrott, 1976). They are in fact clusters of these follicles. Similar in form and function to lymph nodes they are thus sites of antigen encounter for activation and induction of B and T cells and have areas of concentration of each type of lymphocyte. Immigration of naïve B and T cells from primary lymphoid tissues occurs via high endothelial venules which lace the lamina propria. Amplification of both effector and memory cells is another similar function to lymph nodes, resulting in enhanced IgA secretion and cytotoxic killing. Antigen presentation occurs via macrophages and dendritic cells (DC) which have been in close contact with the invaginated basal membrane of M cells. These APCs, especially DC are an integral link between innate and adaptive immunity.

Dendritic cells are APCs of bone marrow origin and are pivotal in directing the immune response in the gut. They exert a disproportionately large effect on T cell function: one

DC may interact with 300-1000 T cells (Stagg et al., 2003). Two major functions of DCs include antigen acquisition and lymphocyte activation. Gut DCs acquire antigen through: 1) connection with M cells, 2) phagocytosis of apoptotic endothelial cells, 3) bacteria which enter the lamina propria through damaged epithelia, and 4) by inserting dendrites between tight junctions into the intestinal lumen. These cellular intelligence operatives are thus exposed to all the extra-cellular environment has to offer. Highly adaptable and sensitive, DCs are capable of not only discriminating sub-molecular variation in antigens, but also of modifying the reaction and differentiation of lymphocytes as a result. For instance, lipopolysaccharide (LPS) from *Escherichia coli* stimulates IL-12 and subsequent T_h1 expansion upon interaction with DCs, while *Porphyromonas gingivalis* derived LPS does not stimulate IL-12 and directs a T_h2 response (Palma et al., 2002). Such fine specificity requires the involvement of precise pattern recognition receptors (PRR). Pathogen associated molecular patterns, or PAMPs, are recognized by a family of cell surface PRRs called Toll-like receptors, or TLRs. First reported by Beutler (2004) and many others since, TLRs are specific for several highly conserved microbial molecules, moieties, and structures. Double stranded RNA (TLR-3), unmethylated DNA (TLR-9), flagellin (TLR-5), and the previously mentioned LPS (TLR-4) are noteworthy examples (Bilsborough and Viney, 2004). The response to TLR recognition of microbial invasion is an intracellular signaling cascade including such molecules as TRAM, TRIF, MyD88, IRAK-1, TRAF-6 and ultimately the transcription factor, NF kappa beta. The products of genes activated by NF kappa beta include the pro-inflammatory cytokines TNF, IL-1, and IL-6 thus stimulating innate

immunity. In addition, NF kappa beta also which functions in the expression of MHC, IL-2, Il-12 and IFN gamma: all of which are molecules important to the adaptive response (Zhang and Ghosh, 2001).

Adaptive Immunity in the Gastrointestinal Tract

One may not think of the adaptive immune system as a barrier in the sense that the physical structure of the GIT and its associated cells of the innate system are, but nonetheless, it is. The adaptive system must be bypassed, broken through or compromised to allow a sustained infection to occur. In this system, lymphocytes of both the B and T variety are selected by antigen and activated. This process is then followed by expansion of the immune cells and hopefully, elimination of the infection. Finally, these cells undergo negative feedback and return to a "resting" state (Abbas and Lichtman, 2003). This entire procedure is done in concert with the previously described cells of the innate response. Mesenteric lymph nodes and Peyer's patches are sites of antigen encounter for lymphocytes in the gut. Antigen recognition and proper co-stimulation result in up-regulation of cytokines such as IL-2, IL-4 and IFN gamma which in turn regulate differentiation of naïve T cells to T_h1, T_h2, and CTL subsets. Decreased expression of CCR7 by T cells and a concomitant increased expression by B cells cause migration out of their respective areas in lymphoid tissue towards each other at the follicular edge. Here B-T interaction culminates in B-cell maturation, and differentiation into effector or memory cells. Secretion of IgA is the primary function of

GIT B cells. All three barriers must interact to ward off the constant potential infections in an environment so conducive to microbial growth. As alluded to earlier, one mystery of this system is that it must also recognize and not fight off bacteria that are at the least not harmful, and very often beneficial, to the host.

Intestinal Microbes

Constructive conjunctive symbiosis*: an association between two different organisms, with bodily union between them, which is of benefit to the physiologic processes of one (or both)[a] of them.* MerckSource 2005 [a] Authors addition

The so called commensal bacteria, those that live within the GIT without harming the host are, I believe, more accurately described by the definition given above. Human beings are in fact, by number of cells, about 90% microbial with 10^8 to 10^{12} bacterial cells/g of intestinal content. The gut microflora have even been described as a metabolic organ (Savage, 1986). Fermentation by hindgut microbiota provides volatile fatty acids, of which butyrate is the primary metabolic substrate for intestinal epithelia (Roediger, 1980). Acetate is ultimately used by peripheral tissues and propionate provides a substrate for hepatic gluconeogenesis. Energy from plant structural carbohydrates, otherwise not available to animals, is obtained in this manner. Coprophagy is practiced by some animal species, allowing the re-capture of nitrogen from bacterial growth in the hindgut. Additionally, the B vitamins biotin, folic acid, and pantothenate, as well as vitamin K are synthesized by resident bacteria. Aside from these more well known

functions, intestinal microflora also participate in many important physiologic interactions with the host.

Research with gnotobiotic or germ-free animals has elucidated numerous benefits derived from intestinal prokaryotes. Such work has indicated that short-chain fatty acids of microbial origin stimulate intestinal blood flow, epithelial proliferation, and gene expression. Germ-free animals exhibit decreased GIT development and epithelial turnover as compared to their microbe inhabited cohorts. Intestinal bacteria stimulate healthy development of gut immune function. For example, as opposed to typical lymphoid tissue, the normal state of Peyer's patch follicles includes chronic germinal centers; the result of constant stimulation by enteric antigens (Cebra, 1999). As a member of the common mucosal immune system, a fully functional gut has implications for immunity in other parts of the body. Bacteria are also known to achieve cell-cell communication both between and across species and thus regulate not only the activity of the colony but favorably influence reactions from the host (Henke and Bassler, 2004). A recent discourse by Sperandino (2004) reviewed the subject of bacterial cross-talk with the endocrine and neuroendocrine systems. Bacterial growth is stimulated by mammalian hormones and cytokines (Burton et al., 2002). Blalock (2005) has referred to the immune system as the 6^{th} sense. Could it be that resident microbes are more than just opportunistic bystanders in this process? Perhaps they are constructive conjunctive symbionts?

One way that the balance between distress and eustress can be manifest is through the population of GIT Microbes. As previously discussed, "good" bacteria are key players in a healthy gut; they deny space to potentially harmful species, digest structural carbohydrates, and provide essential vitamins. An imbalance in the microbial species composition in the GIT is implicated in such maladies as irritable bowel syndrome (Guyton and Hall, 2000). Stressed animals may exhibit marked differences in diet selection and intake, passage rate, digestion, and absorption. Some bacteria are substrate specific, others are generalists. Changes in any one of the previous factors could result in hindgut substrates very different from that found in non-stressed animals, and accordingly, very different microbial populations.

In his review, Tannock (1996) cites numerous monogastric animal studies in which various stressors induce changes to normal microbiota. Tannock (1996) also states that two bacterial populations are particularly sensitive to diet-induced stress: *lactobacilli* usually decrease under stressful conditions while *Escherichia coli* usually increase. Maternal separation in rhesus monkeys resulted in lowered fecal *lactobacilli* and increased susceptibility to infection (Bailey and Coe, 1999). Pigs subjected to early weaning, acute cold, or mixing with non-littermates exhibited increased fecal shedding of *E. coli* compared to controls (Jones et al., 2001). Long-term transport in cattle has also been reported to increase fecal shedding of *E coli.* (Bach et al., 2004). Similarly, transport-induced stress resulted in increased shedding of *Campylobacter spp.* in broiler feces (Whyte et al., 2001). Bacteria constitute 30-50% of fecal material in ruminants

and up to 90% in poultry; monitoring changes in the fecal population of bacteria in response to stress could provide a method to quantify stress in free-ranging birds and animals.

Fecal Near Infrared Reflectance Spectroscopy

Near infrared reflectance spectroscopy (NIRS) involves the detection of light in the near infrared (NIR) band (~800 to 2500 nm) that has been reflected by a substance of interest. When irradiated by this light, organic bonds, primarily CH, OH, and NH, begin to vibrate at characteristic frequencies corresponding to those found within the NIR band. Light at wavelengths of similar frequencies are absorbed by the bond, dissimilar wavelengths are reflected. This occurrence results in a biochemical "snapshot" of the material, via summary of the relative population of bonds present and their detected absorbance and reflection of NIR light. The use of NIRS is widespread in agriculture and natural resource management and one specific application, fecal NIRS, has particular utility for investigations into the nutritional ecology of herbivores (Foley et al., 1998; Stuth et al., 1999). Fecal analysis has obvious advantages as a non-invasive means of obtaining information from free-ranging animals. Samples are relatively easy to obtain and this method allows the animals to go about their daily routines and not be subjected to movement, gathering, and handling. The dry matter in feces is largely bacteria, undigested food, sloughed endothelial cells, digestive secretions and metabolized endocrine products (Church, 1979). Thus, feces are a repository of

physiological information, fraught with opportunity for scientific exploitation. Quantifying nutritional parameters seems a logical primary application of the NIRS technology and indeed, diet quality in several species of livestock and wildlife has been determined (cattle: Coates, 1998; Lyons and Stuth, 1992; Purnomoadi et al., 1998; Ksiksi et al., 2000; Gibbs et al., 2002; goats: Leite and Stuth, 1995; sheep: Krachounov et al., 2000, Li et al., 2006; white-tailed deer: Showers et al., 2006; elk: Brooks et al., 1984; Keating et al., 2004). Coates (1998) and Tolleson et al., (2002) have employed fecal NIRS to predict dry matter intake in ruminants. Dietary tannin content (Tolleson et al., 2000) as well as intake of pine needle (*Pinus ponderosa,* Kronberg et al., 1998), leafy spurge (*Euphorbia esula*, Walker et al., 1998) and mountain big sagebrush (*Artemisia tridentata*, Walker et al., 2000) have been determined with this technique. The use of NIRS allows the researcher to bypass traditional chemistry once a calibration equation is established, thus NIRS is a faster, less expensive technique in the long run.

In addition to diet quality, fecal NIRS has been used to discriminate between fecal samples according to physiological characteristics such as: sex and species in cervids (Tolleson et al., 2005) or sex and reproductive status in cattle (Tolleson et al., 2000, 2001) and sheep (Godfrey et al., 2001). Discrimination between samples from pen-fed animals with and without an external parasite burden has been reported (Tolleson et al., 2006), and Teel et al., (2004) have similarly used NIRS of feces to distinguish between samples from grazing cattle with divergent parasite loads. The mechanism/s by which physico-chemical differences induced by stress or physiological status are expressed in

feces, is not yet known. Stress-mediated changes in digestion, absorption, immunity, and microbiota all seem possible. Monitoring any or all of these via blood or feces would fall under the aforementioned field of bio-forensics.

One hypothesis for the divergent fecal NIR spectra noted above is that tick-induced stress causes changes in the endocrine/immunologic/metabolic homeostasis of the animal which in turn affects host/microbial relationships in the hindgut. Stress responses in the animal could alter intake, diet selection, passage rate and absorption. Additionally, the gut-associated innate immune system could be altered by endocrine changes due to stress. Soderholm et al., (2002) reported increased gut inflammation in chronic stressed versus non-stressed rats. Additionally, degradation of the brush border membrane occurred in surgically stressed rats (Prabhu et al., 2000). The responses listed here all have an endocrine or immune system connection and in fact, direct communication between host and bacteria has been shown to occur through stress related endocrine factors (Sperandino et al., 2003). Tick related stress should also result in disrupted homeostasis and thus alter the host/bacterial ecology. Research conducted in free-ranging animals is needed so that we can better understand and quantify the effects of tick-induced stress on microbial ecology of the hindgut.

Tick Attachment and Feeding

The tick life cycle consist of four stages: 1) egg 2) larvae 3) nymph 4) adult. A single blood meal is taken during each of the latter three stages. One-host ticks feed on a single host species through each of the three post-embryonic stages, while multiple-host ticks such as *A. americanum* drop off the current host animal between stages and subsequently seek another. Mating of adults takes place on the host while attached and feeding. Oviposition by the female occurs after detachment, and the cycle starts again. It is obviously the attachment and feeding by ticks that have detrimental effects on the host.

Attachment is initiated by insertion of the hypostome. This appendage has many hook-like projections which inhibit removal of the attached tick. Additionally, ixodid or hard-bodied ticks, secrete a cement-like substance produced by the salivary glands which helps to hold the feeding tick in place. This cement consists primarily of proteins and glycoproteins but also contains lipids (Kemp et al., 1982). After attachment, formation of a feeding lesion occurs. Small blood vessels rupture and drain into the lesion. Normal response to such an injury, namely platelet formation, plasma coagulation cascade, and vasoconstriction, would suffice to stop the hemorrhaging. Tick saliva, however, contains anti-haemostatic factors affecting all three processes. Platelet aggregation inhibitors found in tick saliva include apyrase (Ribeiro, 1987), moubatin (Waxman and Connally, 1993), disagregin (Karczewski et al., 1994), variabilin (Wang et al., 1996), and prostaglandins (Bowman et al., 1996). Anticoagulation factors in tick

saliva operate through inhibition of either serine protease factor Xa, or thrombin (Bowman et al., 1997). The most well known anticoagulant present in tick saliva is TAP, or tick anticoagulant peptide (Bowman et al., 1997). Anticoagulation serves purposes in tick feeding other than the obvious role of maintaining blood flow through the feeding lesion; it also keeps blood circulating through mouthparts and gut tissues. Ticks thus concentrate the blood meal and excrete approximately 33 - 50% of the fluid intake back into the host (Bowman et al., 1997). Vasodilation also serves to promote blood flow at the feeding site. Prostaglandins, in addition to inhibition of platelet aggregation, are potent vasodilators. When prostaglandin synthesis is impaired, engorged tick weights can be reduced up to 30% (Madden et al., 1996).

Tick-induced Immuno-suppression

In addition to anti-haemostatic factors, tick saliva also contains many other pharmacologically active compounds which suppress inflammation and the immune response, both acquired and innate (Nuttall, 1998). All ixodid species posses immunoglobulin binding proteins (Wang and Nuttall, 1994). As the name suggests, these proteins bind to host antibodies, preventing the subsequent binding to epitopes within the tick. Antibodies are then passed through the tick digestive system and returned to the host. Extracts from tick salivary glands have been demonstrated to suppress human natural killer cell (Kubes et al., 1994) and mouse interferon α (Hajnicka et al., 1998) activity. Decreased natural killer cell function could also lead to reduced

production of interferon, a component of the cytokine cascade involved in directing the T helper cell response (Wikel and Bergman, 1997). Immuno-modulation by the tick is obviously self-serving, but it is also the mechanism which facilitates tick-borne disease transmission.

Saliva activated transmission (SAT) factors (Nuttall and Jones, 1991) are secreted into the skin lesion during feeding. Though SATs are produced by both infected and non-infected ticks (Nuttall, 1998), and function to affect the reaction of skin in the immediate area of the feeding lesion, they also facilitate pathogen transmission to the host and between ticks. For instance, a virus may enter the skin at the feeding lesion and go undetected due to the aforementioned effects of tick saliva constituents. Here in the skin the virus may infect Langerhans cells and be carried to a lymph node where naive lymphocytes are activated (Austyn, 1992) and return to the skin, attracted to tick affected areas. At these locations, uninfected ticks are exposed to the virus. While discerning vector mediated effects on pathogen transmission is not the focus of the proposed research, much of the work done concerning immuno-modulation by ectoparasites has been aimed at that objective. The mechanisms discovered in such research would also be expected to affect overall immune function in the host. If said mechanisms can be adequately characterized and quantified, they could be utilized in the development of bioassays to determine the effects of ticks and or other stressors on livestock.

CHAPTER II

PLANE OF NUTRITION BY TICK BURDEN INTERACTION IN CATTLE: EFFECT ON GROWTH AND METABOLISM

Introduction

Ticks are external parasites which pose a significant economic burden to domestic animal agriculture worldwide (Drummond 1987; Pegram et al., 1991, Meltzer and Norval; 1993, Kivaria, 2006). Direct economic effects of ticks include loss of body weight and condition, lowered reproduction, and hide damage (Barnard, 1985; Teel et al., 1990). Additional hardships may result from disease transmission (Brossard and Wikel, 1997; Kivaria 2006). Combating an external parasite burden may be a considered a "cost of fitness" which incurs a drain on available energy (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Demas, 2004). Protein-energy malnutrition has metabolic, endocrine and immune consequences especially with respect to parasitism (Hughes and Kelly, 2006). The effects of ticks may thus be exacerbated during periods of low nutrition such as those encountered during drought. Many grazing animals worldwide face periods of drought as a common occurrence.

Diet quality and quantity interact to provide the nutritional environment for grazing animals. Within a given plane of nutrition, energetically costly events such as lactation (Bell, 1995) or infection (Elsasser et al., 2000) affect an individuals profile of metabolic

indicators such as glucose, ketones, nitrogen, or fatty acids. These profiles can be used to monitor the extent or duration of such events. Dairy cows challenged with lipopolysaccharide to simulate a short-term inflammatory state had increased blood glucose and decreased non-esterified fatty acids and beta-hydroxtbutyrate compared to those not challenged (Waldron et al., 2006). Bulls experiencing transport stress exhibited higher blood glucose and lactate than non-transported bulls (Chacon et al., 2005). Gupta et al., (2005) observed increased albumin, urea and non-esterified fatty acid concentrations in blood of cattle repeatedly re-grouped and moved to new pens versus non treated controls. It is not known how plane of nutrition and tick burden interact to affect metabolism in cattle. Tick species such as *Dermacentor albipictus* infest cattle during winter months and thus would be confounded with a low nutritional state in many geographic regions. Similarly, adult Lone Star ticks (*Amblyomma americanum*) feed during late spring into summer, a time period often corresponding to drought in the southern Great Plains of the US, and thus lower forage quantity and quality.

Physiological effects of ticks on cattle have been investigated and this work has produced varied results in observed physiologic characteristics. The observed variation could be due to differences in the species and number of ticks employed in each study or to differences in individual animal responses to the ticks. For instance, Willis et al., (1995) reported that 20 to 120 pairs of *A. americanum* caused elevated heart rates in growing beef steers but did not affect temperature, respiration, intake, or any metabolic indicators in blood. Riley et al., (1995) observed higher respiration rates of yearling beef

steers infested with 25 pairs of *A. maculatum* than those with 0 or 75 pairs. In the same study, creatine kinase concentrations and white blood cell counts were higher for tick-infested steers than controls. In a 2-year pasture study (grazing period = 8 weeks each year), *A. maculatum* infested Hereford steers averaged 8.21 kg less than control animals in year 1 and 12.42 kg less in year 2 (Williams et al., 1978). In both years, significant increases occurred in total serum protein, serum globulin, and plasma fibrinogen, and decreases occurred in the albumin/globulin ratio of infested animals. These authors also noted less ticks attaching over time. In a study utilizing Holstein-Friesian cows exposed to gradually increasing burdens of *Boophilus microplus,* control cows produced 2.86 l more milk and 0.14 kg more butterfat per day and gained 10.6 kg more body weight than infested cows over a 15-week period (Jonsson et al., 1998). The daily DMI of control cows in this study was 0.83 kg greater than that for infested cows by week 12. Seebeck et al. (1971) reported that daily intake was decreased approximately 15 % and gain by approximately 50% in cattle infested with *B. microplus* versus tick free cattle. Tolleson et al., (2004) reported that DMI of cattle on a moderate quality diet was reduced in tick versus non-tick cattle during peak tick feeding. Neither IGF-1, glucose, lactate or cortisol were affected by tick treatment in that study, but within the tick treated group, each of these constituents was related to the rate at which tick feeding progressed in individual animals. The objective of this current research was to examine the plane of nutrition by tick burden interaction in cattle, and to determine the effects of this interaction on physiological indicators of growth and metabolism.

Materials and Methods

The experiment was conducted at the Texas A&M University Animal Science Teaching, Research, and Extension Center. All animal procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Eight month old Angus cross steers (n = 28, 194 ± 3.0 kg) obtained from the Texas Department of Criminal Justice commercial cow herd were stratified by pre-trial weight and DMI into one of four groups (n = 7 per group) in a 2 x 2 factorial arrangement. Catgories were: moderate (14.0 ± 1.0% CP, 60 ± 1.5% TDN) versus low (7.0 ± 1.0% CP, 58 ± 1.5% TDN) plane of nutrition, and control (no tick) versus tick treatment (300 pair of adult *A. americanum* per treated animal). Both the moderate and low diets were cottonseed hull based with various proportions of cottonseed meal, corn, sorghum and a vitamin-mineral premix to achieve the desired plane of nutrition. All animals were fed the moderate diet for 28 days during which they were gentled and acclimated to Calan Gate® feeders. Steers were then individually fed their respective experimental diets *ad libitum* and intake was monitored for 35 days prior to and 21 days following the start of tick infestation (day 0). Non-shrunk weights and BCS were obtained on day -35, -7, and 21. Animals were housed outside in concrete-floored pens (6.0 x 10.0 m) and individually fed from day -35 to day -7. At this point the animals were moved inside where they were housed and fed in 1.0 x 2.5 m stanchions through the end of the experiment. Due to the configuration of the stanchions in the room, seven replicates of four animals each, one per tick treatment by

plane of nutrition group were stratified across the stanchion room. Water was provided *ad libitum*. Stanchions were cleaned daily.

Ticks used in this study originated from research and teaching colonies maintained at the Texas A&M University Department of Entomology Tick Research Laboratory. Experimental populations of *A. americanum* were originally established and periodically supplemented with progeny of gravid female ticks collected from livestock residing at the Texas Agriculture Experiment Station in Sutton county Texas, and at the Hill Ranch in Edwards county Texas. While being reared for the study, tick colonies were maintained within separate glass humidity chambers where environmental conditions approximated 20^{o}C, 90% relative humidity, and 14:10 light:dark photoperiod. The feeding cycle for this species consists of infestation followed by 7 days of location and attachment by female ticks, then intense feeding beginning at about day 10 until engorgement and drop-off at approximately 14 days. A period of predominately male feeding occurs from day 14 to 17. All feeding is complete by day 21. Environmental conditions within the stanchion room were maintained at 21 ± 1.0^{o} C, approximately 50 % relative humidity and a 14:10 light:dark photoperiod.

Blood samples were harvested via coccygeal venipuncture on days -7, 0, 7, 8, 9, 10, 11, 13, 17 and 21 and processed to yield plasma. Cortisol concentrations were determined in a single RIA from duplicate samples using a single antibody RIA procedure (Carroll et al., 2006) and utilized: rabbit anti-cortisol antiserum (Pantex, Div. of Bio-Analysis

Inc., Santa Monica, CA, Cat. #P44) diluted 1:2500; standards made by serial dilution (8000 pg/100 mL to 3.9 pg/100 mL) of 4-pregnen-11ÿ,17,21-triol-3,20-dione (Steraloids Inc., Newport, RI, Cat. #Q3880-000); and radio-labeled cortisol: 3H-Hydrocortisone (1,2-3H, NEN, Boston, MA, Cat. #NET-185). Counts per minute (cpm) obtained from a liquid scintillation spectrophotometric beta-counter (Beckman Coulter LS 6500) and unknown cortisol concentrations were calculated using Assay Zap software (Biosoft, Cambridge, UK). Cortisol antiserum cross-reactivity: corticosterone, 60%; deoxycorticosterone, 48%; progesterone, 0.01%; and estradiol, 0.01%, (determined by Pantex). Intraassay CV was 8.0%. Aliquots of serum were assayed in duplicate to determine the concentration of IGF-I by RIA (Strauch et al., 2003). The final dilution of the primary antibody was 1:120,000 and the goat anti-rabbit secondary antibody was used at a dilution of 1:60. The IGF-I antibody utilized was AFP4892898 anti-hIGF-I (A. F. Parlow, National Hormone and Peptide Program, Torrance, CA). Recombinant IGF and radiolabeled (125I) were purchased from Peninsula Labs (San Carlos, CA). All samples were assayed in a single assay with an intra-assay CV of 7.8%. Urea nitrogen (BUN), glucose (GLU), non-esterified fatty acids (NEFA), beta-hydroxy-butyrate (BHBA), albumin (ALB), hemoglobin (HGB), gama-glutamyl transpeptidase (GGT), and aspartate aminotransferase (AST) were determined by colorimetric assays at the Texas A&M Veterinary Medical Diagnostic Laboratory.

Differences between groups for all constituents were determined by GLM procedures in SAS. Main effects were tick treatment and plane of nutrition. Treatment, plane of

nutrition, and the treatment by plane of nutrition interaction were tested with the replicate by treatment by plane of nutrition interaction. Tukeys adjusted LS means were used for mean separation. Stepwise multiple regression (Steel and Torrie, 1980) was employed to determine relationships between growth and endocrine or metabolic constituents. Chi-square procedures (Steel and Torrie, 1980) were applied to detect differences in proportion of samples from treatment groups belonging to specified categories. Simple linear regression (Steel and Torrie, 1980) identified relationships between metabolic constituents.

Results

The tick feeding cycle progressed normally with engorged ticks commencing drop off on day 9, peaking at day 12 and terminating by day 17 (Figure 1). Tick feeding was not affected by host plane of nutrition. Cattle visibly experienced some degree of irritation as evidenced by periodic attempts to groom the ticks from their backs. DMI (% BW) was greater ($P < 0.05$) in moderate (3.79 ± 0.06) than low (3.36 ± 0.04) and also in control (3.69 ± 0.04) than tick treated (3.46 ± 0.05) animals. Within plane of nutrition, DMI was greater ($P < 0.05$) in control than treated animals for both the moderate (3.94 ± 0.05 versus 3.65 ± 0.06 respectively) and low (3.45 ± 0.04 versus 3.26 ± 0.04 respectively) groups. When expressed as a proportion of mean pre-treatment (day -7 to day 0) DMI, overall DMI was depressed during the peak tick feeding / blood sampling period (Figure 2). All cattle gained weight throughout the trial (Figure 3). Treatment

did not affect BW. There was no difference in BW (kg) between the plane of nutrition groups on day -35 (192.8 ± 5.5 versus 196.5 ± 6.0), but on day -7 and day 21, the moderate steers weighed more than the low steers (244.1 ± 8.7 versus 227.7 ± 8.4, $P < 0.07$; and 283.4 ± 8.0 versus 244.0 ± 7.9, $P < 0.001$; respectively).

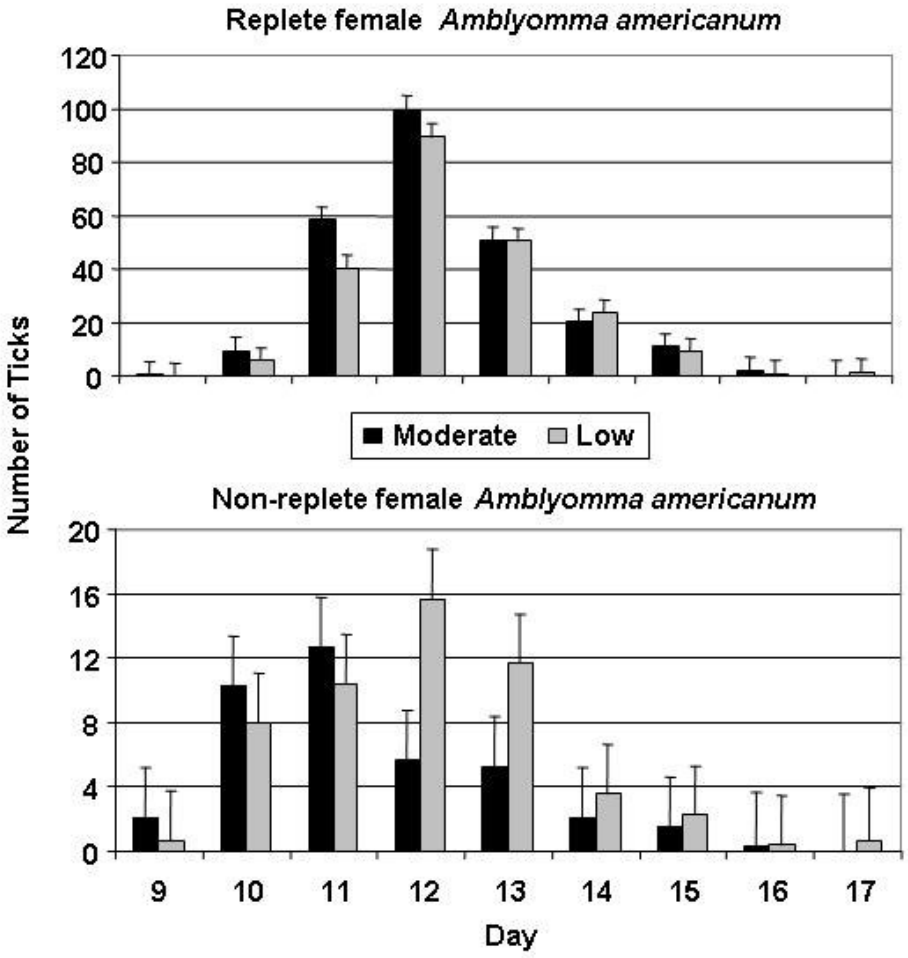

Figure 1. Daily drop-off of fully fed (replete) and partially fed (non-replete) female *Amblyoma americanum* by plane of nutrition in growing beef steers.

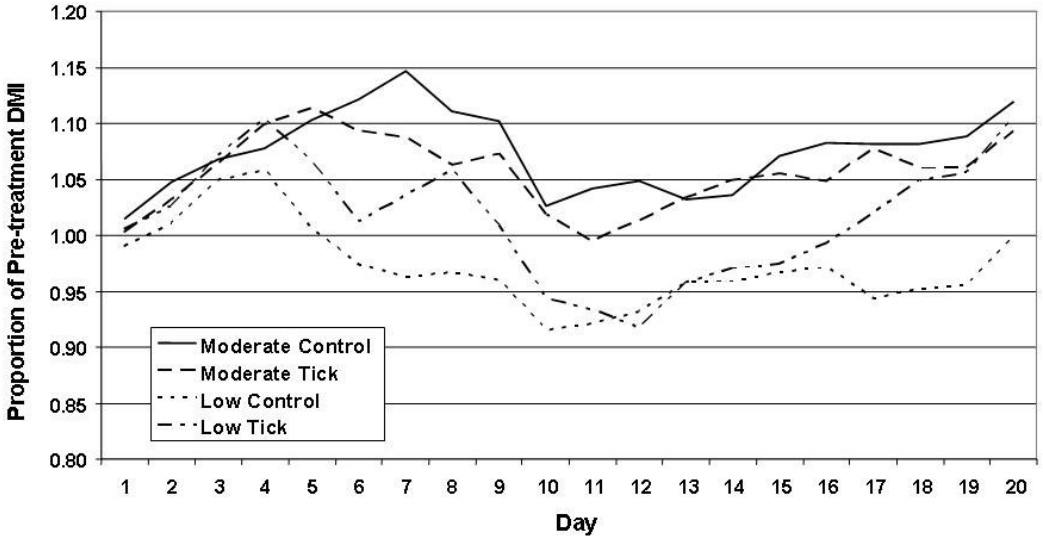

Figure 2. The effect of plane of nutrition and tick-treatment on dry matter intake (% body weight basis) as a proportion of mean pre tick-treatment dry matter intake by day in growing beef steers.

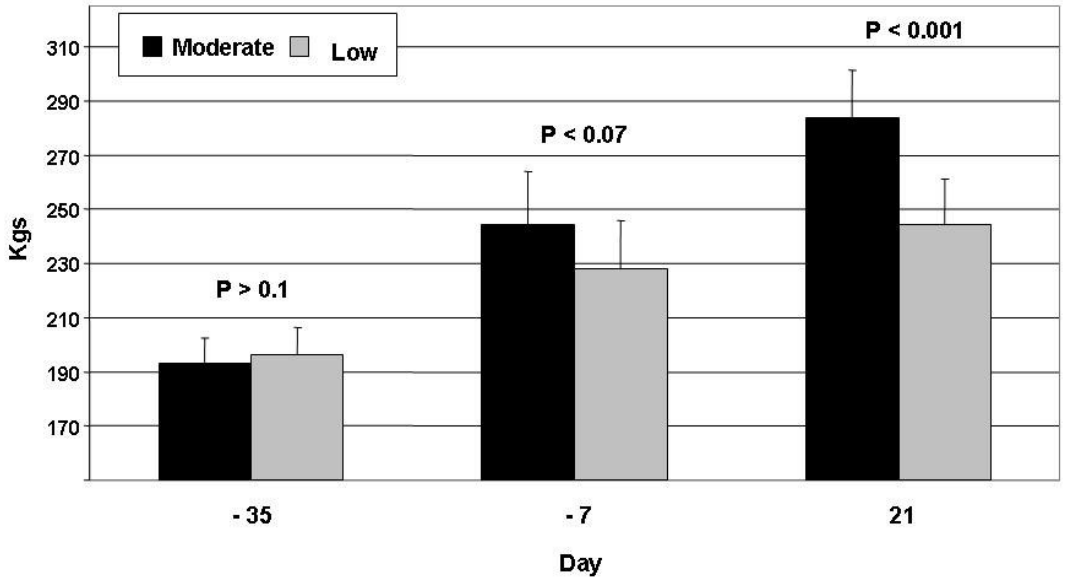

Figure 3. The effect of plane of nutrition and tick-treatment on body weight by day in growing beef steers.

On day -35 BCS (1 = thin, 9 = fat) averaged 4.5 ± 0.1 for both nutrition groups but by day 21, BCS was 5.5 ± 0.1 and 5.0 ± 0.2 for moderate and low respectively ($P < 0.05$). Likewise, treatment did not affect BCS. Values for ADG (kg d^{-1}) between day 0 and 21 were greater ($P < 0.001$) for moderate (1.31 ± 0.15) versus low (0.54 ± 0.06) steers. Treatment did not affect ADG.

All blood constituents are reported in least square means (Table 1). Cortisol (ng/ml) was affected by plane of nutrition and treatment at the $P < 0.08$ level. There was also a significant day effect in that day 21 (12.65 ± 0.85) was higher ($P < 0.05$) than day 0 through 17 (~ 7.8) but not day -7 (10.36 ± 0.85). Figure 4 represents plasma cortisol concentrations by plane of nutrition and treatment. Within the moderate plane of nutrition, tick treated cattle had higher ($P < 0.05$) plasma cortisol than non-treated. Day 21 samples (12.31 ± 1.11) within the moderate group also exhibited higher ($P < 0.05$) cortisol concentrations than any other day (all < 8.8). There was no treatment effect within the low plane of nutrition but day 17 cortisol concentration (6.76 ± 1.29) was lower ($P < 0.05$) than day 21 (12.93 ± 1.29). IGF-1 (ng/ml) was greater ($P < 0.01$) in moderate than low animals and in ($P < 0.03$) control compared to tick treated animals (Figure 5). Within the moderate group, there was no difference in IGF-1 between treatments. However, within the low group, IGF-1 was greater in control ($P < 0.05$) than in treated steers. During peak tick feeding, IGF-1 was greater ($P < 0.05$) in controls than tick treated animals (day 10: 175.6 ± 9.7 versus 141.5 ± 9.7; day 11: 179.4 ± 9.7 versus

Table 1. The effect of plane of nutrition and tick-treatment on metabolic and endocrine constituents in growing beef steers.

	Plane of Nutrition				Treatment			
	Moderate		Low		Control		Tick	
Constituent	LSMean	SE	LSMean	SE	LSMean	SE	LSMean	SE
Albumin	3.51	0.01	3.34	0.01	3.4	0.01	3.45	0.01
Urea Nitrogen	7.02	0.08	2.89	0.09	5.01	0.09	4.9	0.09
Glucose	101.49	0.88	90.44	0.9	96.27	0.89	95.27	0.89
BHBA [a]	454.6	10.02	445.92	10.17	428.18	10.1	472.34	10.11
NEFA [b]	0.25	0.01	0.26	0.01	0.24	0.01	0.27	0.01
ASAT [c]	63.03	0.75	63.03	0.76	61.05	0.76	65.01	0.76
GGTP [d]	30.06	0.98	20.83	1.0	16.14	0.99	34.75	0.99
Hemoglobin	27.75	1.14	26.41	1.43	27.74	1.42	26.42	1.42
IGF-1 [e]	191.53	2.44	125.34	2.51	169.71	2.47	147.16	2.47
Cortisol	7.36	0.37	9.61	0.38	7.34	0.37	9.63	0.38
	Plane of Nutrition by Treatment							
	Mod/Con		Mod/Tick		Low/Con		Low/Tick	
	LSMean	SE	LSMean	SE	LSMean	SE	LSMean	SE
Albumin	3.48	0.02	3.53	0.02	3.32	0.02	3.37	0.02
Urea Nitrogen	7.19	0.12	6.85	0.12	2.83	0.12	2.96	0.12
Glucose	100.08	1.24	102.89	1.26	93.23	1.27	87.65	1.26
BHBA [a]	425.82	14.06	483.37	14.26	430.54	14.44	461.3	14.34
NEFA [b]	0.26	0.01	0.25	0.01	0.22	0.01	0.3	0.01
ASAT [c]	63.25	1.06	62.83	1.07	58.85	1.08	67.21	1.08
GGTP [d]	45.19	1.38	14.93	1.4	24.32	1.41	17.35	1.4
Hemoglobin	31.86	1.98	23.64	2.01	23.62	2.03	29.19	2.02
IGF-1 [e]	200.26	3.42	182.81	3.47	139.17	3.56	111.5	3.53
Cortisol	6.02	0.52	8.69	0.53	8.66	0.54	10.57	0.54

[a] Beta-hydroxtbutyrate

[b] Non-esterified fatty acids

[c] Aspartate aminotransferase

[d] Gama-glutamyl transpeptidase

[e] Insulin-like growth factor 1

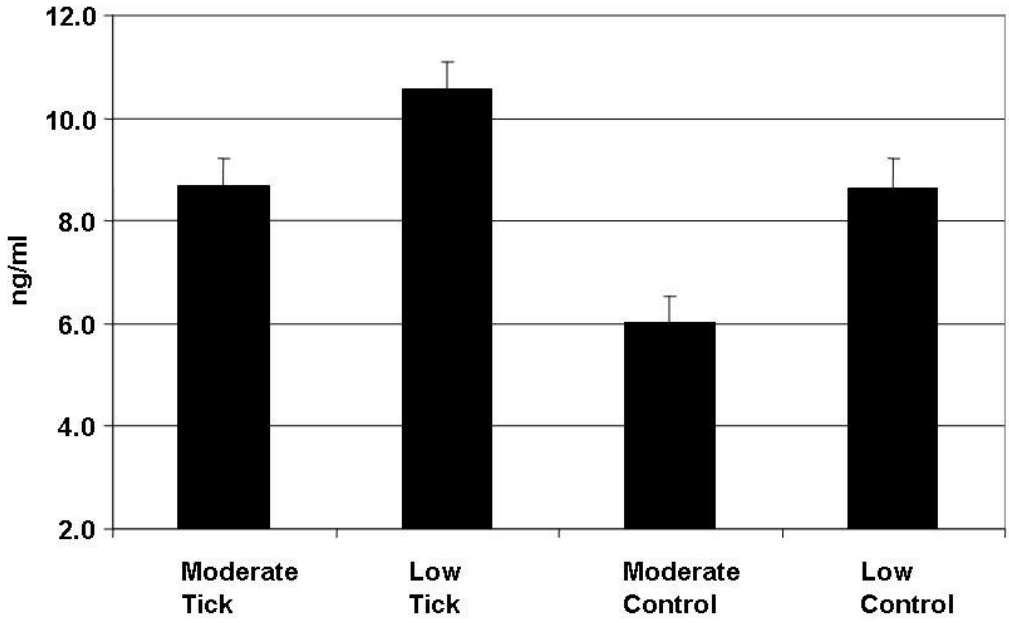

Figure 4.The effect of plane of nutrition and tick-treatment on plasma cortisol concentration in growing beef steers.

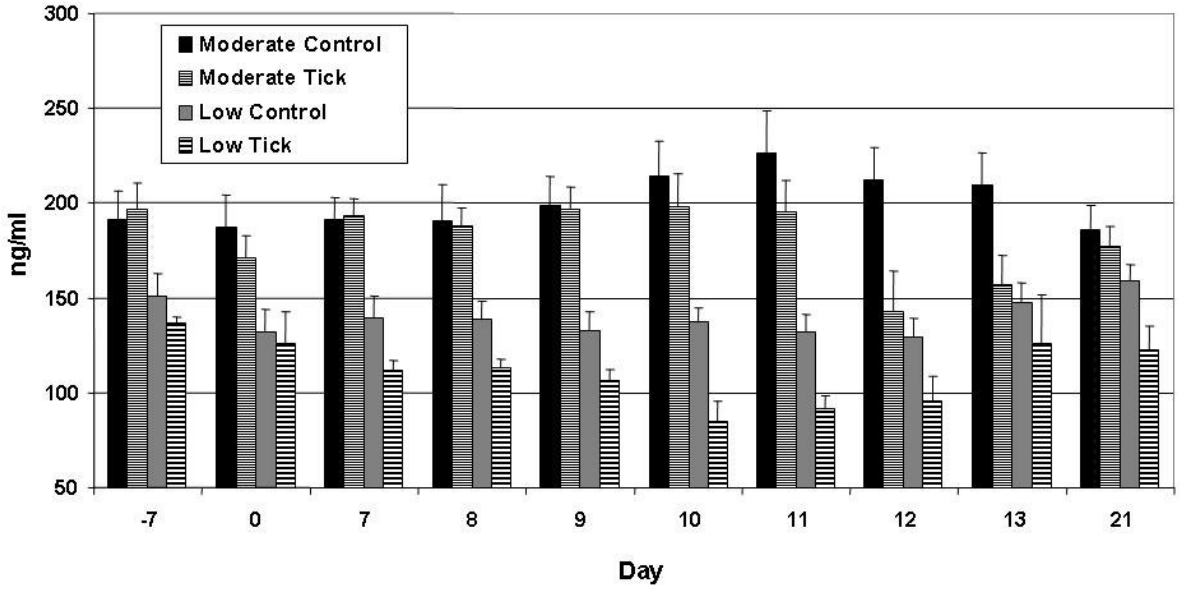

Figure 5. The effect of plane of nutrition and tick-treatment on plasma IGF-1 concentration by date in growing beef steers.

143.5 ± 9.7; day 12: 170.2 ± 10.2 versus 118.3 ± 9.9; and day 13: 179.4 ± 10.2 versus 146.5 ± 10.5). Treatment had no affect on any of the metabolites measured in this study. Plane of nutrition affected ($P < 0.02$) ALB, BUN and GLU in that values from moderate group animals were higher than those from the low group. The effect of day was significant ($P < 0.01$) for all metabolites except GLU ($P < 0.1$) and approached significance ($P = 0.09$) for GGT. Within plane of nutrition, tick burden did not affect any constituent. A model containing BUN, GLU, and NEFA at day 12 and 21 described 54.9, 66.4, and 75.5 % of the variation in day 21 BCS, day 21 BW and day 0 to day 21 ADG ($P < 0.01$).

Discussion

The ticks employed in this study were able to successfully complete their feeding cycle and were not affected by plane of nutrition. In a review of the relationships between ectoparasites and herbivore nutrition, Sutherst (1987) reports that an effect of host nutrition on ticks has been observed by several authors. For instance, he cites a study in which "lean" sheep had a 50 % higher number of ticks than "fat" sheep. Additionally, growing steers fed lucerne exhibited greater resistance to *B. microplus* than did steers grazing autumn/winter pasture in Queensland (Sutherst et al., 1983). The cattle fed lucerne were heavier than the pastured group at the end of the feeding trial (~ 400 versus 300 kg, respectively) and maintained a greater tick resistance than the original pastured cattle after returning to pasture for the following year. Perhaps the current study was not

of sufficient duration to allow expression of an effect of host nutritional status on tick viability. As noted previously, the cattle in this study did experience irritation from the ticks as evidenced by attempted grooming behavior, and although not quantified, did not appear to be different for either plane of nutrition.

DMI was apparently affected by ticks but could also have been due to the coincident frequent blood sampling procedures since a depression in DMI also occurred for control cattle. Daily DMI in pen-fed animals appears to cycle normally (Schwartzkopf-Genswein et al., 2004; Cooper et al., 1999; Caldeira et al., 2007) but the decrease observed here is similar to that observed in a previous tick burden study in our laboratory (Tolleson et al., 2004). In both studies, animals were individually fed experimental diets in outdoor pens for 35 days prior to being placed in stanchions indoors. In the previous study, blood samples were obtained on days -7, 0, 10, 14, 21, 28, and 35 relative to tick treatment. So there was less frequent blood sampling during the peak tick feeding period. It seems unlikely that in both studies, a decrease in DMI would have occurred at the same time period relative to tick treatment if this was just part of a normal cycle. In both studies, cattle generally increased intake throughout the adaptation period. Additionally, steers in the previous study generally increased DMI from day 21 to 35.

Seebeck et al., (1971) reported lower intake in cattle fed a lucerne-based diet (14.9 % CP) due to repeated infestations with *B. microplus*, as compared to a tick-free *ad libitum*

fed group. Also in their study was a tick-free group of cattle which were pair-fed to the same level of intake as the tick group. Both of these groups gained less weight than the tick-free group with *ad libitum* intake. After being treated with acaricide and placed on pasture, the previously tick infested cattle exhibited less compensatory gain than the pair-fed group. Byford et al., (1992) summarized the effects of ectoparasites on cattle and report a 32% reduction in ADG due to the Gulf Coast tick (*A. maculatum*). All cattle in our study gained weight, even those in the low plane of nutrition / tick treated group. As expected, moderate plane of nutrition cattle gained more and were in better condition at the end of the trial than the low group.

We did not monitor intake or gain in the current study past day 21. We also did not have a non-tick treated, non-blood sampled experimental group. Anecdotally, however, there were six extra steers from the original group not used in the study but maintained in dry lot consuming (*ad libitum*) the moderate experimental diet plus any orts from the experimental cattle on both diets during the time period of the feeding trial. These non-experimental animals weighed 171.50 ± 9.65 kg on day -35 (versus 197.88 ± 3.71 kg for the moderate control steers) but were 265.02 ± 10.83 kg by day 21 of the experiment. Corresponding day 21 BW for the moderate control steers was 283.4 ± 8.0 kg. Thus proportionally, the extra cattle gained numerically more than did the moderate control treatment group (56 versus 45 % increase in BW). At the other extreme, the low plane of nutrition, tick treated group achieved a 22% increase in BW.

The cortisol concentrations observed in this study would indicate that these animals were indeed under stress. Grandin (1997) reports in a summary of several studies that baseline cortisol concentration for cattle is < 9 ng/ml. Our individual values ranged from 1.43 to 28.73 ng/ml. Both low plane of nutrition and tick burden increased plasma cortisol. High cortisol levels have been associated with malnutrition (Douyon and Schteingart, 2002). Dairy cows fed straw had higher cortisol concentrations than those fed straw and silage (Odensten et al., 2007). Willis et al. (1995) using *A. maculatum* and Riley et al. (1995) using *A. americanum* reported no effect of ticks on cortisol in cattle. The Byford et al., (1992) review reported an increase in cortisol coincident with increasing numbers of horn flies (*Haematobia irritans*). In our study, moderate control cattle possessed the lowest cortisol concentrations (6 ng/ml) and these lie within the baseline values reported by Grandin (1997). Conversely, low plane of nutrition tick treated cattle had the numerically highest cortisol values (10.6) in the experiment and this value is similar to that reported by Grandin (1997) for animals in the "restraint in headgate" category. All animals in this study were restrained in a headgate within their individual feed stanchions during blood sampling.

Our observed magnitude of range in cortisol values is similar to that observed before (~ 5 ng/ml) and after (~ 12 ng/ml) jugular catheterization in cattle (Stewart et al. 2007). Cortisol concentration was similar between the low control and moderate tick groups. Of the highest quartile of plasma cortisol values (15.78 ± 0.53 ng/ml, n = 60) obtained between day 0 and 21 in this study, 61 % were from low plane of nutrition cattle, 64 %

from the tick groups and 41 % from peak tick feeding / blood sampling days (10 to 14), as compared to 32, 43, and 42 % respectively from the lowest quartile (2.80 ± 0.13 ng/ml, n = 60). It is interesting to note that while the proportion of samples belonging to the upper and lower quartiles differed ($P < 0.05$, $X^2_{,2}$) with respect to plane of nutrition and treatment, the proportion of samples with respect to day did not.

Our observation that the low nutritional plane animals had lower IGF1 than their better nourished counterparts is not surprising. When beef heifers fed to either maintain BCS or lose condition until becoming anovulatory were compared, the maintain BCS group had higher plasma IGF1 than the heifers which lost condition (Bossis et al., 1999). Mean concentration of IGF1 sequentially increased from low to moderate and tick to control in the current study (Figure 4). These values closely mirror those reported by Caldeira et al., (2007) in groups of sheep with BCS (1 = thin; 5 = fat) of 1.25, 2.0, 3.0, or 4.0. Corresponding IGF1 (ng/ml) values were 70.0 ± 13.5, 156.8 ± 27.6, 173.9 ± 27.7, and 209.0 ± 14.7 for each BCS group respectively. Disruption to homeostasis also related to IGF1 levels. Disease induced IGF1 reduction in bull calves (125 kg BW) was highly correlated to the magnitude of infection and to decreased BW gain (Elsasser et al., 1998). These authors also observed that hepatic mRNA for GH receptor and IGFI was decreased in infected calves. In a previous study conducted by our laboratory, hepatic GH receptors were greatest in non-tick treated steers, less in tick-treated steers, and least in steers pair-fed to match the tick treated animals, on day 14 of the *A. americanum* feeding cycle (Tolleson et al., unpublished data). No differences in plasma IGF1 was

observed between these treatment groups. IGF1 was considered to be the best indicator of short term metabolic status and body composition in humans undergoing intense physical exertion and caloric restriction (Nindle et al., 2007). Although cortisol was related to both tick treatment and nutritional status in the current study, with respect to the combination of parasitism and sub-optimal nutrition, IGF1 was the most highly indicative constituent measured.

The metabolites measured in this study were not by affected by tick treatment. Perhaps the immunosuppressive capabilities of the ticks (Nuttall,1998) attenuated the normal host pro-inflammatory response. In our aforementioned tick burden study, haptoglobin was not different in tick treated versus non-tick treated or pair-fed steers, and in fact was highest on the day of experimental tick infestation than on day 10. Cattle infested with *B. microplus* possessed less serum ALB than non-infested animals (O'Kelly and Kennedy, 1981). These authors found no effect of ticks on either NEFA nor GLU. O'Kelly et al. (1971) observed reduced HGB but not NEFA or GLU in *B. microplus* treated compared to no-treated steers. In *A. americanum* infested cattle, Willis et al., (1995) report no effects of tick feeding on cortisol, total blood protein, BUN or GLU. It should be noted that in their study, a lighter tick burden (20 to 120 adult pairs per animal) was utilized than in our study (300 adult pairs per animal).

Plane of nutrition caused expected differences in ALB, BUN, and GLU, but surprisingly not in NEFA or BHBA. We also did not observe the negative relationship between GLU

and NEFA often reported for nutritionally stressed animals (Kida, 2002; Bossis et al., 1999; Yelich et al., 1996; van Knegsel et al., 2005). A nutritional balance analysis (Nutbal Pro, Stuth et al., 1999) indicates that the low plane of nutrition animals would have been just slightly positive for energy, and the reader will recall that these animals did gain BW throughout the experiment. There were some interesting relationships observed between metabolic and endocrine constituents measured. For instance, BHBA was negatively correlated with GLU within both the moderate ($P < 0.04$) and low ($P < 0.01$) groups. IGF1 and GLU were positively correlated in both planes of nutrition (moderate, $P = 0.1$ and low, $P < 0.003$). Lastly, the NEFA versus cortisol relationship was positive in both the moderate ($P < 0.03$) and low ($P < 0.0006$) groups. These relationships are to be expected within the context of energy metabolism as affected by level of nutrition and hypothalamic-pituitary-adrenal axis activity. Restraint and isolation for 2 to 6 hours increased cortisol and glucose but not NEFA in treated steers versus controls (Apple et al., 2005). Kushibiki et al., (2003) observed that NEFA and cortisol increased in response to TNF alpha versus saline in lactating cows. The increase in NEFA occurred even though these early lactation cows (i.e. in a negative energy balance) already exhibited elevated NEFA. These same authors report that IGF1decreased in the TNF alpha challenged cows.

In an attempt to elucidate the relationships between tick burden, plane of nutrition and animal performance, multiple stepwise regression models were developed. The most notable of these was one that contained BUN, GLU, and NEFA at day 12 and 21. This

model described approximately 50 to 75 % of the variation in BCS, BW and ADG at the end of the trial. Individual animal metabolic responses within the treatment groups were thus as important as were treatment differences. Tick burden affected various characteristics of growth and metabolism in these cattle and was exacerbated by a low plane of nutrition; but in addition to the individuality observed, experimental day had a large effect in this study. It would appear that both tick burden and the combined stress of the experiment had an effect on animal performance, though it would be difficult to distinguish the effects of ticks versus those of confinement and blood sampling from this data. Based on our results and those of previous workers, our hypothesis is that the stress of tick feeding was confounded with that of frequent blood sampling. In a companion to this paper, we discuss the need for alternate methods of evaluating stress and metabolism in livestock. Fecal chemistry or near infrared spectroscopy both offer potential solutions for non-invasive monitoring of grazing animals.

CHAPTER III

PLANE OF NUTRITION BY TICK BURDEN INTERACTION IN CATTLE: EFFECT ON FECAL CHEMISTRY

Introduction

Stress is a somewhat overused and ambiguous term as popularly applied. Stress by definition is a real or perceived threat to homeostasis but may have several manifestations. Miller et al., (1994) separate stress into acute, episodic acute, or chronic categories. Acute stress invokes the classic "fight or flight" response via activation of the hypothalamic-pituitary-adrenal axis (HPA). Once the stressor has been eliminated or resolved to the satisfaction of the individual, physiologic indicators of stress such as increased glucocorticoids, catecholamines, heart rate or blood pressure return to pre-stress levels. The ability to react to a stressful situation by specifically directing resources toward immediately important functions and away from those less important, is vital to self-preservation. The effects of cortisol, a glucocorticoid, on energy metabolism are well documented. Increased energy substrates are mobilized for brain and muscle function as well as for immune responses when needed. Overstimulation of the HPA may be detrimental, however, due either to catabolism (Christiansen et al., 2007) or immunosuppression (Venkatraman and Pendergast, 2002). Episodic acute or chronic stress may thus have a quite different physiology than short-term events.

Sopolsky (2003) has reported on the long-term effects of stress on overall health and well-being.

Ticks are external parasites which cause loss of body weight and condition, lowered reproduction, and hide damage in cattle (Barnard, 1985; Teel et al., 1990). Ticks are also a significant vector of disease (Brossard and Wikel, 1997; Kivaria 2006). Tick burdens are stressful to the animal parasitized; various effects on host physiology have been documented (Willis et al., 1995; Riley et al., 1995; Williams et al., 1978; Jonsson et al., 1998). Mounting a defense against an external parasite burden incurs a drain on available energy (Sheldon and Verhulst, 1996, Lochmiller and Deerenberg, 2000, Demas 2004). Ticks may invoke responses similar to inflammation and thus have been reported to depress intake and performance (Seebeck et al., 1971). Protein-energy malnutrition has metabolic, endocrine and immune consequences especially with respect to parasitism (Hughes and Kelly, 2006). The effects of ticks may thus be exacerbated in animals concomitantly experiencing poor nutrition.

Effective management of ticks on grazing animals would be facilitated by accurate non-invasive methods of detection. Feces offer potential as a material to provide information about animal health and nutrition in a non-invasive manner. Numerous authors have explored this possibility, for example, Lysyk et al., (1985) found that moisture and nitrogen content of cattle manure changed with season of grazing (apparent diet quality). Holecheck et al., (1982) found similar relationships in grazing cattle. Steers grazing

dormant native prairie and supplemented with corn had lowered fecal pH and ADF content than non-supplemented steers, but higher fecal organic matter output (Bodine and Purvis, 2003). Etheridge et al., (1984) observed that diet composition affected fecal moisture, VFA's and pH in pigs. Diet quality and quantity obviously affect fecal composition, stress may do likewise. Stress causes changes in metabolism which can be manifest in fecal chemistry. Both cold and restraint stress increased fecal output and moisture over that recorded for non-stressed rats (Barone et al., 1990). These effects were additive. Transport stress affected not only cortisol but immunoglobulin A (IgA) content in the feces of reindeer (Rehbinder and Hau, 2006). Bacteria constitute a large percentage of fecal dry matter in ruminants (Merchen, 1988; Van Soest, 1982). Mason (1969, as quoted in Lyons and Stuth, 1992) observed that bacterial cell concentrations increased in sheep feces as the diet became less fibrous. Products of bacterial metabolism also appear in feces and may be present in sufficient quantity to affect overall fecal chemistry (Lyons and Stuth, 1992). In a review of numerous monogastric animal studies, Tannock (1996) indicates that various stressors induce changes to normal fecal microbiota. Tannock (1996) also states that two bacterial populations are particularly sensitive to diet-induced stress: *lactobacilli* usually decrease under stressful conditions while *Escherichia coli* usually increase. When pigs were subjected to early weaning, acute cold, or mixing with non-littermates they exhibited increased fecal shedding of *E coli* as compared to non-stressed controls (Jones et al., 2001). Long-term transport has also been reported to increase fecal shedding of *E coli.* in cattle (Bach et al., 2004) and of *Campylobacter spp.* in broilers (Whyte et al., 2001). Maternal

separation in rhesus monkeys resulted in lowered fecal *lactobacilli* along with increased likelihood of infection (Bailey and Coe, 1999). Human response to changes in diet (via enteral feeding) has been observed to include altered fecal microbial populations and short chain fatty acid composition (Whelan et al., 2004).

Tick burdens have been reported to affect fecal chemistry as detected by near infrared reflectance spectroscopy (NIRS) in cattle (Tolleson et al., 2007a). This previous study was designed to examine the ability of NIRS in feces to detect tick burdens but did not attempt to characterize any differences in fecal chemistry analyzed by traditional reference methods. It is unknown which fecal constituents are affected by tick burdens, thus resulting in alterations to absorption of near infrared light. Several characteristics may be affected by stress due to ticks. Changes in passage rate might be indicated by differences in DM or OM. Bacterial populations could be affected by changes in substrate due to differences in digestion. Both of these may cause a change in pH or production of VFA's. Changes in immune function could be reflected in differences in IgA. Fecal cortisol has been utilized as a non-invasive indicator of stress (Mostle and Palme, 2002), perhaps measurement of this hormone would be useful for monitoring tick burdens as well. The objective of the current research was to determine the effects of plane of nutrition and tick burdens in cattle and is one of a three-part series. Specifically this report contains results of the potential interaction between ticks and nutrition on fecal chemistry.

Materials and Methods

The experiment was conducted at the Texas A&M University Animal Science Teaching Research and Extension Center. All animal procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Growing Angus cross steers (n = 28, 194 ± 3.0 kg) were stratified by pre-trial weight and DMI then assigned to one of four treatments (n = 7 per group) in a 2 x 2 factorial arrangement: moderate (14.0 ± 1.0% CP, 60 ± 1.5% TDN) versus low (7.0 ± 1.0% CP, 58 ± 1.5% TDN) plane of nutrition, and control (no tick) versus tick treatment (300 pair of adult Lone Star ticks (*Amblyomma americanum*) per treated animal). Both experimental diets were cottonseed hull based. All animals were fed the moderate diet for 28 days during which they were gentled and acclimated to the Calan Gate feeders. Steers were individually fed the moderate and low diets *ad libitum* for 35 days prior to and 21 days following the start of tick infestation (day 0). Animals were housed outside in concrete-floored pens (6.0 x 10.0 m) and individually fed in Calan Gate® feeders from day -35 until day -7 at which point they were moved inside to be housed and fed in 1.0 x 2.5 m stanchions thereafter. Seven replicates of four animals each, one per treatment group were stratified across the stanchion room. Water was provided *ad libitum*. Stanchions were cleaned daily. Ticks used in this study originated from research and teaching colonies maintained at the Texas A&M University Department of Entomology Tick Research Laboratory. Tick rearing conditions are reported in (Tolleson et al., 2007b). The feeding cycle for this species consists of infestation followed by 7 days of location and attachment by female

ticks, then intense feeding beginning at about day 10 until engorgement and drop-off at approximately 14 days. A period of predominately male feeding occurs from day 14 to 17. All feeding is complete by day 21.

Fecal samples were collected at approximately 0700 each day. On day -7, 0, 7, 10, 14, 17, and 21 fecal samples were divided into A and B aliquots, approximately 200 g wet weight each. Aliquot A was used fresh for determination of pH and culture of *Lactobacillus spp.* and *Escherchia coli* (Gerhardt et al. 1994), the remainder of aliquot A was stored at -20 C and later used for determination of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate via HPLC (Chen and Lifschitz, 1989). Aliquot B was stored at -20 C and later thawed, dried overnight at 60 C in a forced air oven then ground to 1mm particle size in a laboratory mill. Once processed, aliquot B was used to determine DM and OM via standard AOAC (1990) procedures. This aliquot was also used for determination of fecal IgA via ELISA procedures (Goñi et al., 2005). A sub-sample of aliquot B was sent to the Reproductive Physiology laboratory at Mississippi State University for determination of fecal cortisol (Bowers et al., 2005). Actual extraction technique was adapted from the methods described in Wasser et al., (1994) in which 0.5 g of dried feces was placed into 5 ml of 80% methanol and vortexed for ~14 h. The vortexed samples were then centrifuged at 2500 rpm for 15 min. Supernatant was then taken and evaporated under air for 12-14 hrs. The pellet was then reconstituted in 0.5 ml of 80% methanol for RIA determinations. The representative corticoid content of feces was determined based on Cortisol and Corticosterone RIA. Data obtained from the

assays were then converted into ng/g feces on a dry matter basis. The fecal corticoid values are concentrated due to ~82.4% moisture removal when dried at 60°C. Though not reported as part of this manuscript, it is important to note that blood samples via coccygeal venipuncture were also collected on day -7, 0, 7, 10, 14, 17, and 21 relative to tick treatment. Differences between treatments for all constituents were determined by GLM procedures in SAS. Main effects were treatment and plane of nutrition. Treatment, plane of nutrition, and the treatment by plane of nutrition interaction were tested with the replicate by treatment by plane of nutrition interaction. Tukeys adjusted LS means were used for mean separation. Stepwise multiple regression (Steel and Torrie, 1980) was employed to determine relationships between tick numbers and fecal constituents. Simple linear regression (Steel and Torrie, 1980) identified relationships between fecal constituents.

Results

Tick feeding cycle was as expected (Figure 6) and was not affected ($P > 0.1$) by plane of nutrition. Fecal constituents are reported as least square means. Low plane of nutrition resulted in increased ($P < 0.05$) fecal DM (28.55 ± 0.20 %) versus the moderate group (26.96 ± 0.21 %). Day also affected ($P < 0.02$) fecal DM in that day 21 (26.91 ± 0.43 %) DM was less than that recorded on day -7 (28.73 ± 0.38 %). There was a plane of nutrition by day interaction ($P < 0.08$) for fecal DM (Figure 7). In the low group, fecal DM was increasing toward the end of the trial, while in the animals receiving the

moderate diet, fecal DM was decreasing toward the end of the trial. Fecal OM was similarly affected ($P < 0.001$) by plane of nutrition in that the low group had a higher value than the moderate ($94.68 \pm 0.11\%$ versus $93.52 \pm 0.12\%$ respectively). Day also affected ($P < 0.001$) fecal OM with the lowest values observed on days -7, 0, and 21 (< 93.9 %) and highest between day 7 and 17 (> 94.4 %). There was a plane of nutrition by day interaction ($P < 0.004$) for fecal OM (Figure 8). In the low animals, fecal OM was higher in the post tick feeding period than in the pre-feeding period. In the moderate animals, fecal OM was low on day -7 and day 21, intermediate on day 0 and higher during the tick feeding period. Only day affected ($P < 0.004$) fecal pH (Figure 9). Post tick feeding pH was generally lower than pre-feeding and was lowest on day 17. There were no observed differences in pH among and between plane of nutrition or treatment groups.

Treatment had no effect ($P > 0.1$) on any VFA's measured. Day, however, affected ($P < 0.05$) all VFA's measured in that generally speaking, VFA concentrations were lower at the end of the trial than at the beginning. Plane of nutrition affected ($P < 0.003$) all but isovalerate, for all others the moderate group was higher than the low group. There were significant (propionate, $P < 0.1$, all others $P < 0.01$) plane of nutrition by day interactions for all VFA's measured.

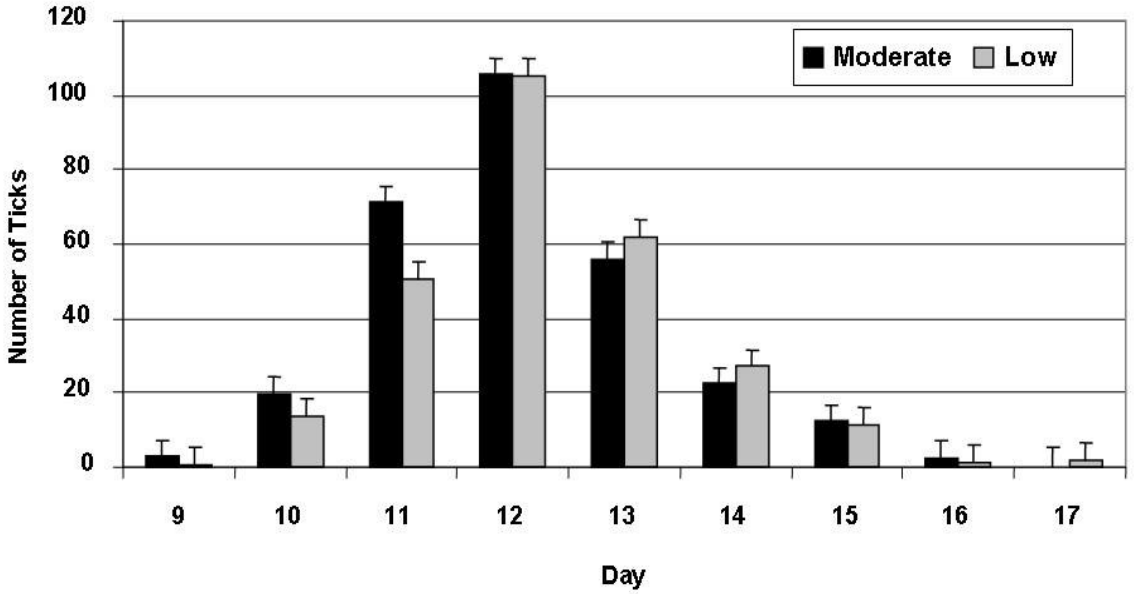

Figure 6. Daily drop-off of total female *Amblyoma americanum* by plane of nutrition in growing beef steers.

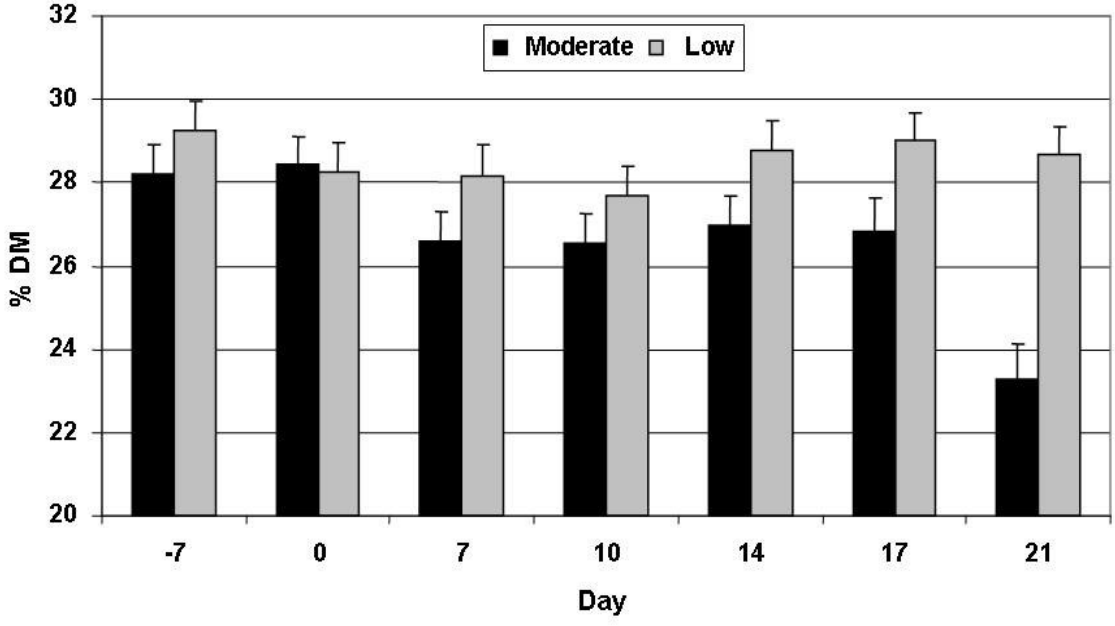

Figure 7. The effect of plane of nutrition on fecal dry matter by day in growing beef steers.

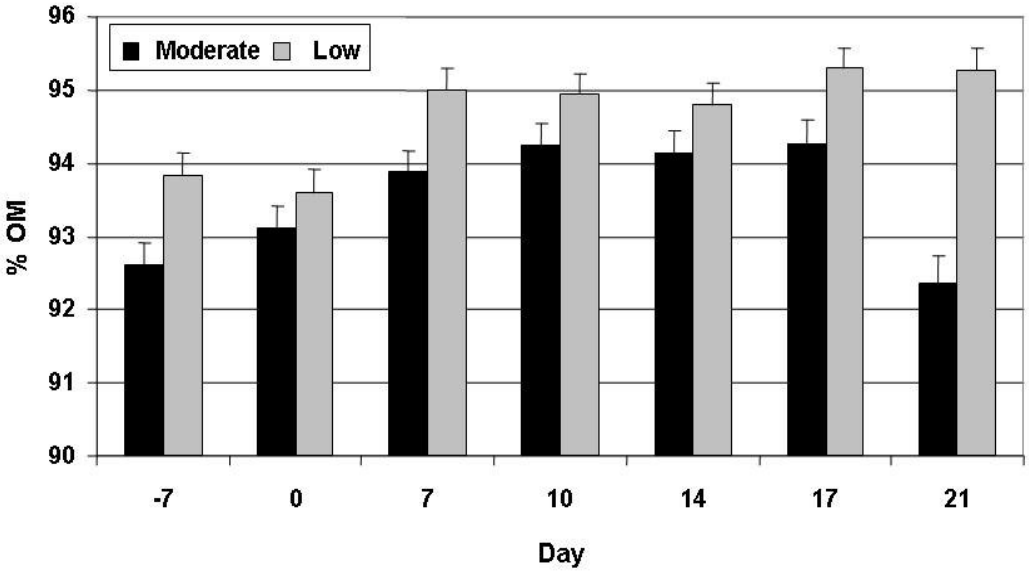

Figure 8. The effect of plane of nutrition on fecal organic matter by day in growing beef steers.

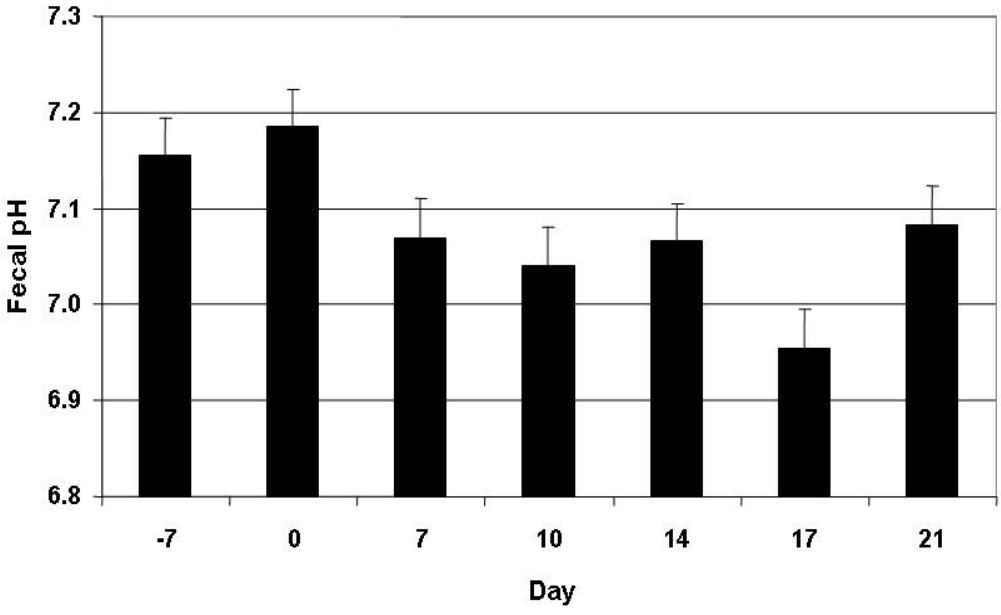

Figure 9. The effect of day on fecal pH in growing beef steers.

Within the low group, VFA concentrations declined sharply at first then slowly, while within the moderate plane of nutrition, VFA concentrations rose slightly then declined (e.g. acetate, Figure 10). Treatment affected (P = 0.09) fecal cortisol (ng/ml) with the control animals having lower (3.90 ± 0.22) concentrations than tick treated animals (4.83 ± 0.22). Day affected (P = 0.001) fecal cortisol (day 13 = 6.24 ± 0.38, others ranged from 3.28 ± 0.39 (day 6) to 4.73 ± 0.39 (day 9)). Plane of nutrition had no effect (P > 0.1) on fecal cortisol (moderate 4.48 ± 0.22, low 4.26 ± 0.22). Plane of nutrition did affect (P = .002) IgA (mg/ml). Higher values were recorded for the moderate group (0.0033 ± 0.0001) than for the low group (0.0026 ± 0.0001). Again, day affected (P = 0.001) fecal IgA concentrations as day 10 to 17 were higher than all others. There was a plane of nutrition by day (P = 0.09) interaction (Figure 11) due to different concentrations of fecal IgA on day -7. There was no affect (P > 0.1) of treatment.

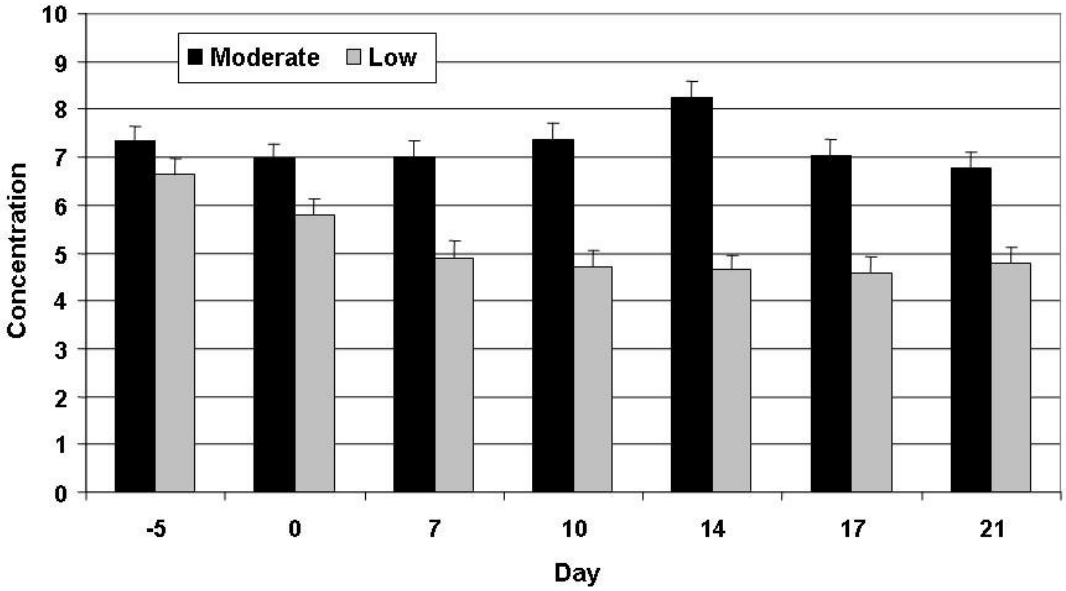

Figure 10. The effect of plane of nutrition on fecal acetate concentration by day in growing beef steers.

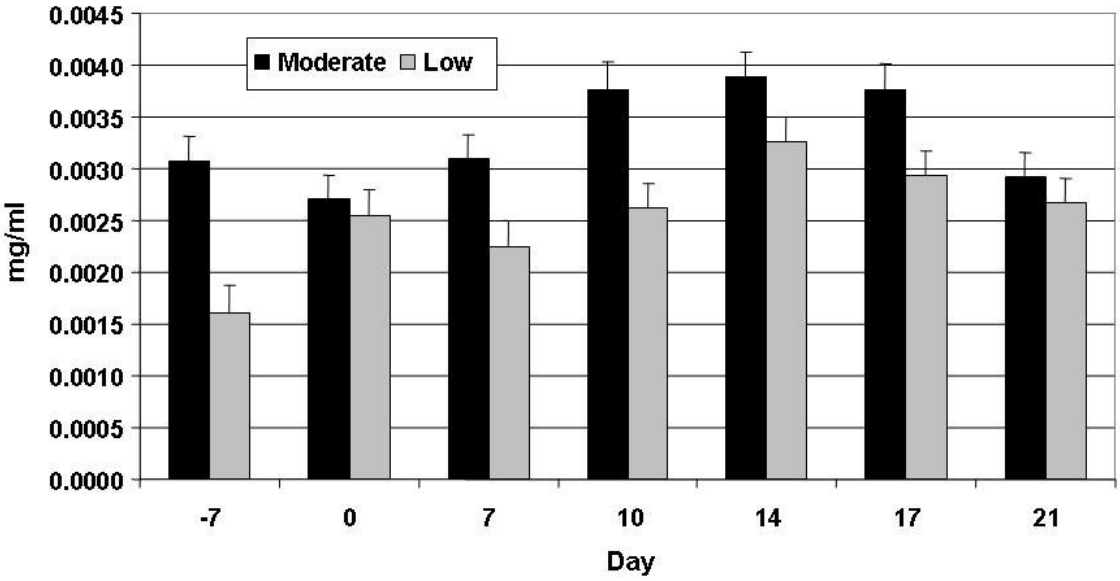

Figure 11. The effect of plane of nutrition on fecal immunoglobulin A concentration by day in growing beef steers.

Tick group values were 0.0030 ± 0.0001, and for controls, 0.0029 ± 0.0001.Neither plane of nutrition nor treatment affected ($P > 0.1$) the number of colony forming units of *E. coli* or *Lactobacillus spp.* cultured from fecal samples. Day affected both bacterial species (*E. coli* $P < 0.02$, *Lactobacillus* $P < 0.04$), in that values were lowest on day 17 to 21 (< 75 CFU) than on day -5 and 0 (> 112 CFU) for *E. coli*, and similarly for *Lactobacillus*, day 7 (65 CFU) was lower than all other days (> 109 CFU). Within the moderate plane of nutrition, there were no significant fecal constituents on day 10 and 14 to describe daily female cumulative tick drop numbers. However a model containing only cortisol described 58.7 and 61.9 % of the variation in daily total and replete female ticks respectively. In the low plane of nutrition, a model including cortisol, propionate, isovalerate and DM at day 10 and 14 described 95.4 % of the variation in total female

ticks. Similarly, a model containing cortisol, propionate, *E. coli*, and DM accounted for 94.9 % of replete female tick drop counts.

Discussion

Plane of nutrition resulted in differences in fecal DM and OM. This is to be expected and agrees with Lysyk et al., (1985) who found that the fecal moisture of cattle grazing South Dakota cool season pastures was greatest in June and declined throughout the summer. The highest moisture content occurred simultaneously with the highest nitrogen content, i.e. during active plant growth as opposed to mature dormant forage. The gradual increase in fecal DM observed during and after the tick / sampling stress period agrees with observations made in Sky Lab astronauts (Holdman et al., 1976). Similar to the inverse relationship between diet quality and fecal DM, fecal OM content will be greater in a relatively lower digestibility ration than in a higher digestibility ration (Huston and Pinchak 1991), even though prediction of diet quality by means of fecal chemistry indices may be problematic (Leite and Stuth, 1990). Although tick treatment had no effect on OM in this current study, the general increasing trend in these constituents during the intense tick feeding / blood sampling period would agree with the finding by Tolleson et al., (2002) that fecal NIRS predicted DOM in beef heifers fed a pelleted ration was lower during peak feeding periods of an *A. americanum* infestation than during non-peak feeding periods. It should be noted that in the previous study there were no non-tick treated animals, comparisons were made between six, 3 to 4 day

segments of the 21 day tick feeding cycle. Also in contrast to the current study, the previous animals were not subjected to blood sampling procedures and so this additional stressor was not confounded with tick feeding. Due to our experimental design, clear distinctions can not be made between the effects of tick feeding and blood sampling in the current research. As discussed in part one of this series (Tolleson et al., 2007b), DMI was lower during the peak tick feeding / blood sampling period in both this current study and in a previous study by our laboratory (Tolleson et al., 2004). In the previous study, blood sampling was less frequent, occurring on day 10 and 14 only. Thus lower DMI, higher DM and OM, and the presence of two stressors seems hardly coincidental. Add to this the observation in the current study that fecal pH was not affected by plane of nutrition or treatment but was lower in the tick feeding as opposed to pre-tick feeding period. It is not known why, in the entire sample population, lower pH occurred at the same time as lower VFA concentrations, this is opposite of the expected relationship (Barajas and Zinn, 1998; Canh et al., 1998; Van Baale et al., 2004). Perhaps other H^+ donors were present but not measured. Fecal pH decreases in concert with the amount of starch present in feces (Wheeler and Noller, 1977, Barajas and Zinn, 1998) and increases in concert with protein (Haaland et al., 1982, Barajas and Zinn, 1998). When analyzed within the moderate plane of nutrition however, fecal pH was negatively correlated ($P < 0.01$) with total VFA's ($r^2 = 0.20$), and with acetate ($r^2 = 0.13$), propionate ($r^2 = 0.22$), and butyrate ($r^2 = 0.32$) individually. Within the low plane of nutrition, only propionate had a significant relationship ($P < 0.05$) with fecal pH ($r^2 = 0.04$).

Diet has been shown to affect the shedding of *E. coli* in cattle feces (Van Baale et al., 2004; Russell et al., 2000; Sindt et al., 2000) but with sometimes conflicting results. For instance a diet of grain versus hay will lower fecal pH and result in lower *E. coli* populations. These gram negative organisms may develop acid resistance on a sustained nutrient environment. A short-term dietary shift from grain to hay will then reduce fecal shedding of these bacteria such that this practice has been proposed as a management tool to reduce *E. coli* contamination of beef (Russell et al., 2000). Interestingly, neither diet nor tick burden affected fecal bacterial populations as determined in this study. As with previous constituents, day of the experiment was a contributor to differences in fecal bacteria. *Lactobacillus spp*. numbers were lower in the early stage of tick feeding / blood sampling (day 7), while *E. coli* was reduced in the later stages (day 17 to 21). Even so, we did not observe the inverse relationship between these two bacteria species reported by Tannock (1996). They were in fact somewhat positively related. Within the low group, *E. coli* versus Lactobacillus had an $r^2 = 0.25$ ($P < 0.001$), and a corresponding value of 0.15 ($P < 0.003$) in the moderate plane of nutrition. Fecal pH was not correlated with either species regardless of plane of nutrition. Perhaps the fecal pH values recorded here did not reach a level of acidity (i.e. < 6.0) to favor growth of *Lactobacillus* over *E.coli* (Russell et al., 2000).

Immunoglobulin A is the major antibody secreted by mucosal tissues such as the gastrointestinal tract (Abbas and Lichtman, 2000). Plane of nutrition and day of the experiment affected fecal IgA in the current study while tick treatment did not. The

moderate group had higher antibody concentrations in feces than the low group. This observed effect of nutrition is in agreement with such authors as Fontenla de Petrino et al., (2007) who found that IgA secreting cells were reduced in rats fed a protein restricted diet versus controls. Realimentation only partly recovered IgA secretion in this study. In healthy human males, exercise and energy restriction led to a decrease in salivary IgA that was restored after re-feeding (Oliver et al., 2007). The absence of an observed treatment effect is counter to reports such as Ponferada et al., (2007). These workers found that 6 hr of restraint stress in rats reduced fecal IgA in a 10 day trial, but IgA levels returned to normal after 5consecutive days of restraint treatment. Similarly, in mice subjected to 4 consecutive days of restraint stress of either 1 or 4 hrs duration, fecal cortisol increased in both instances over that of controls, but fecal IgA increased for only the 4 hr treatment (Jarillo-Luna et al., 2007). In this study, adrenalectomy restored fecal IgA production in restraint stressed mice. It is not known why tick feeding did not suppress fecal IgA in these steers. Fecal cortisol concentrations would indicate that these animals were indeed stressed, as would the plasma cortisol values reported in the first paper of this series. Fecal cortisol was in fact the only constituent measured that was affected by treatment and not by plane of nutrition. There was again a significant day effect for fecal cortisol. The highest average daily fecal cortisol observed was for day 13, during peak tick feeding and after six days of repeated blood sampling. While not all of the constituents considered here are practical candidates, of the constituents measured in this study, fecal cortisol offers the best possibility of a non-invasive means of assessing tick stress by way of a chemistry derived method.

CHAPTER IV

PLANE OF NUTRITION BY TICK BURDEN INTERACTION IN CATTLE: DETECTION VIA NEAR INFRARED REFLECTANCE SPECTROSCOPY OF FECES

Introduction

Ticks are an economic burden to the livestock industry (Barnard 1985; Byford et al., 1992). The losses associated with ticks are distributed between lowered weight gain, lowered reproductive success, disease treatment, and hide damage (Teel et al., 1990). The high potential for loss justifies the cost of treatments for ticks (McLeod, 1995). Anti-acaricides are often applied at times convenient to the manager and may not coincide with either an economic threshold of tick burden or an effective timing for tick control. Poorly timed application is especially likely in extensive management situations where handling of animals is infrequent and may be costly or stressful on the animals (i.e. cost outweighs benefit). Ticks and mosquitoes are the most prevalent vectors of emerging and re-emerging diseases in the world (Gubler, 1998). As such they are possible routes of transmission of either natural or intentional infections. Vector control programs have been successful but often short-lived (Gubler, 1998), constant monitoring may be required for sustained control. Physical inspection for ticks requires capture and handling of animals. Surveillance of free-ranging animals would provide a faster and less obtrusive means of detection. Both tick management from a livestock husbandry

standpoint and a biosecurity standpoint would be facilitated by a rapid reliable non-invasive monitoring technique. Feces would be an ideal material to provide information on free-ranging animals if in fact it contained constituents that were related to, or a product of, the tick infestation.

Feces are made up of a variety of metabolic end products. In ruminants the dry matter content of feces is approximately 25%, of this a large proportion (40-60%) is bacteria and bacterial remnants (Church, 1979). The remainder consists of undigested or indigestible foodstuffs, sloughed tissue, digestive secretions and endocrine products (Church, 1979). Fecal chemistry is obviously affected by diet but can also be determined by the physiological state of the individual. Body mass and digestive morphology dictate fecal characteristics such that one species may produce a dry pelleted feces while another may excrete an amorphous semi-solid material. Disease or infection may be manifest in changes to normal fecal constituency (Stonerook et al., 1996), diarrhea being an extreme example. Stress can alter fecal production and passage rate (Barone et al., 1990) as well as composition (Holdman et al., 1976; Jarillo-Luna et al., 2007; Bach et al. 2004) and one would assume the altered nutrient composition is due to changes in exposure of chyme to absorption, or time for hindgut bacterial fermentation among others. Thus if ticks are stressful it seems logical to hypothesize that the actions of ticks might result in measurable changes in fecal chemistry. Collection of feces can be non-invasive thus suited to application in free-ranging animals. If fecal chemistry is related to tick burden and if this could be detected rapidly and accurately, fecal analysis would be

a good method to monitor ticks in grazing livestock especially on rangelands. A rapid reliable non-invasive technique is needed to make this a reality. Near infrared reflectance spectroscopy (NIRS) is one possible means of providing just such a technique.

Near infrared reflectance spectroscopy involves the detection of light (~700 to 2500nm) reflected by a substance of interest. Organic bonds absorb characteristic bands of near infrared (NIR) light and reflect others. Thus an NIR spectrum provides the analyst with a physico-chemical snapshot of that substance. Two substances which differ in physico-chemical properties will produce different NIR spectra. It is this occurrence that has been exploited by scientists to develop predictive calibration models by pairing NIR spectra with reference chemistry. Since NIRS is a rapid, accurate, non-destructive technique, once calibrations have been developed, further chemical determinations can be made relatively quickly and inexpensively as compared to the reference method. The NIRS technique has been applied to numerous agricultural products (Roberts et al., 2004) and of particular interest here, to the determination of dietary (Landau et al., 2006; Walker et al., 2007) or physiological (Tolleson et al., 2005) characteristics of grazing animals. Specifically, fecal NIR spectra have been reported to be different between cattle (*Bos spp.)* and horses (*Equus caballus*) with and without a tick burden (Tolleson et al., 2007a). In addition to different host species, in this report multiple groups of animals, different diets, and several tick species were utilized in a pre-test:post-test design, i.e. no non-tick treated controls were used. A different study by our research group (Tolleson et al., 2004) compared fecal NIR spectra between tick treated and non-treated cattle on a

moderate quality (14%CP, 60% TDN) diet and found results similar to the multiple group study. However, spectra from the non-tick treated animals also contained some differences during the time period when ticks were present on the treated animals. Thus the ability to use fecal NIRS as a practical tool for monitoring tick burdens in livestock remains in question. The study described herein was designed to determine the ability of NIRS to detect differences in feces from animals on two different planes of nutrition and either experiencing or not experiencing a tick burden.

Materials and Methods

The experiment was conducted at the Texas A&M University Animal Science Teaching Research and Extension Center. All animal procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Growing Angus cross steers (n = 28, 194 ± 3.0 kg) were stratified by pre-trial weight and DMI then assigned to one of four treatments (n = 7 per group) in a 2 x 2 factorial arrangement: moderate (14.0 ± 1.0% CP, 60 ± 1.5% TDN) versus low (7.0 ± 1.0% CP, 58 ± 1.5% TDN) plane of nutrition, and control (no tick) versus tick treatment (300 pair of adult Lone Star ticks (*Amblyomma americanum*) per treated animal). Both moderate and low diets were cottonseed hull based. All animals were fed the moderate diet for 28 days during which they were gentled and acclimated to the Calan Gate feeders. Steers were individually fed moderate and low diets *ad libitum* for 35 days prior to and 21 days following the start of tick infestation (day 0). Animals were housed outside in concrete-floored pens (6.0 x

10.0 m) and individually fed in Calan Gate feeders from day -35 to day -7. At this point the animals were moved inside where they were housed and fed in 1.0 x 2.5 m stanchions thereafter. Seven replicates of four animals each, one per treatment group were stratified across the stanchion room. Water was provided *ad libitum*. Stanchions were cleaned daily. Ticks used in this study originated from research and teaching colonies maintained at the Texas A&M University Department of Entomology Tick Research Laboratory. Tick rearing conditions are reported in part one of this series (Tolleson et al. 2007b). The feeding cycle for this species consists of infestation followed by 7 days of location and attachment by female ticks, then intense feeding beginning at about day 10 until engorgement and drop-off at approximately 14 days. A period of predominately male feeding occurs from day 14 to 17. All feeding is complete by day 21. Fecal samples were collected at approximately 0700 each day. Samples were stored at -20 C and later thawed, dried overnight at 60 C in a forced air oven then ground to 1mm particle size in a laboratory mill. Ground samples were re-dried at 60^{o} C for 12 hrs prior to analysis by NIRS. Near infrared spectra were obtained on a Foss® 6500 scanning monochrometer in the 1100-2498 nm range. Spectra were assembled into groups by plane of nutrition, treatment, and by plane of nutrition by treatment. Periods of 7 ± 1 days correspond to significant delineations in the tick feeding cycle. Day -7 to -1 was pre-infestation (period 1). Day 0 to 6 comprised the early feeding stage (period 2) while day 7 to 14 marked the peak female tick feeding period (period 3). Day 15 to 21 consisted primarily of male tick feeding (period 4). Spectra were thus also grouped into these periods for comparison. Combinations of these groupings were created and

analyzed as well. As a further test of the biological significance between spectra from experimental groups, samples were also randomly assembled into groups and subjected to the same statistical methods as the experimental groups. All groups were randomly divided and designated as calibration (~ 75 %) or validation (~ 25 %) sets. Discrimination between groups of fecal spectra was accomplished by 2-block PLS regression (Martens and Martens, 2001) with cross validation within the WinISI® chemometric software package. Differences in proportion of correct identifications between groups were derived by Chi Square procedures (Steel and Torrie, 1980). Principal component score plots (Johnson, 1998) are used to visually illustrate differences in fecal NIR spectra. Calibration for numerical prediction of tick numbers was also performed with the WinISI® product via modified PLS regression (Martens and Martens 2001). Calibration equation evaluation was according to Williams (2005). Briefly, $R^2 > 0.9$ is considered excellent, $R^2 > 0.8$ is good, $R^2 > 0.7$ is adequate, and $R^2 < 0.6$ is poor.

Results

Plane of nutrition had a large effect on fecal chemistry (Tolleson et al., 2007c) and thus NIR spectra. Discrimination was successful ($R^2 = 0.89$, SE of cross validation (SECV) = 0.17) between moderate and low groups of spectra (n = 289 each). Identification of validation samples was also successful for both plane of nutrition groups (96 correct out of 97 samples each). When all treatment group spectra (i.e. tick treated versus control,

day -7 through day 21) were used there was little discrimination (R^2 = 0.13, SECV = 0.48) between them and subsequently less successful validation as well (tick 57% correct and control, 69% correct). Removing the pre-tick infestation samples somewhat improved the discriminant ability between treatment groups. In this case, R^2 = 0.34 and SECV = 0.53. Correct identification of group membership in validation samples was 74% for tick and 61% for controls. Discrimination between treatment groups within plane of nutrition yeilded similar results. Calibration statistics (R^2; SECV) were 0.30; 0.49 for the moderate, and 0.44; 0.49 for the low group. Percent correct identification of group membership in the moderate plane of nutrition was 72 and 65 for control and tick treated respectively. Corresponding values within the low plane were 76 and 61.

Table 2. Discriminant calibration and validation statistics for periods of the tick feeding cycle across both the moderate and low planes of nutrition via near infrared reflectance spectroscopy of feces.

			Validation			Validation		
Calibration	R^2_a	$SECV_b$	Group	N Val. Smpls.	% Correct	Group	N Val. Smpls.	% Correct
Prd. 1 vs 2	0.86	0.26	1	48	0.98	2	48	0.98
Prd. 1 vs 3	0.88	0.20	1	48	1.00	3	55	1.00
Prd. 1 vs 4	0.91	0.20	1	48	1.00	4	42	1.00
Prd. 2 vs 3	0.70	0.34	2	48	0.79	3	55	0.89
Prd. 2 vs 4	0.79	0.35	2	48	0.85	4	42	0.88
Prd. 3 vs 4	0.57	0.39	3	55	0.80	4	42	0.71

a. multiple coefficient of determination

b. standard error of cross validation

Discriminant ability between periods was variably successful and was inverse to the degree of temporal adjacency (Table 2). The comparison between period 1 and 4 resulted in the greatest discrimination (R^2 = 0.91, SECV = 0.20) and success rate for validation of group membership (100 % for both periods), while the period 3 versus 4 discrimination was lowest (R^2 = 0.58, SECV = 0.39) but had moderately successful group identification (80% period 3 and 71% period 4). Discrimination results between periods within both the moderate and low planes of nutrition (Table 3) followed a

Table 3. Discriminant calibration and validation statistics for periods of the tick feeding cycle within the moderate and low planes of nutrition via near infrared reflectance spectroscopy of feces.

Moderate								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Val. Group	N Val. Smpls.	% Correct
Prd. 1 vs 2	0.87	0.28	1	24	0.96	2	23	0.91
Prd. 1 vs 3	0.92	0.19	1	24	1.00	3	28	0.96
Prd. 1 vs 4	0.92	0.22	1	24	0.96	4	20	1.00
Prd. 2 vs 3	0.89	0.34	2	23	0.96	3	28	0.82
Prd. 2 vs 4	0.75	0.39	2	23	0.91	4	20	0.95
Prd. 3 vs 4	0.65	0.40	3	28	0.86	4	20	0.55
Low								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Group	Val. Group	% Correct
Prd. 1 vs 2	0.80	0.34	1	23	0.74	2	23	1.00
Prd. 1 vs 3	0.89	0.18	1	23	1.00	3	28	1.00
Prd. 1 vs 4	0.98	0.19	1	23	1.00	4	22	1.00
Prd. 2 vs 3	0.89	0.26	2	23	0.91	3	28	0.93
Prd. 2 vs 4	0.95	0.26	2	23	0.96	4	22	0.95
Prd. 3 vs 4	0.72	0.37	3	28	0.82	4	22	0.68

$_a$. multiple coefficient of determination

$_b$. standard error of cross validation

pattern similar to that observed for the entire sample set. The same was true for period within treatment group (Table 4). Period 1 versus 4 exhibited the greatest difference in fecal NIR spectra in both planes of nutrition and both treatment groups. Period 3 versus 4, and period 2 versus 3 demonstrated the least discriminant ability in the plane of nutrition, and treatment groups, respectively.

When comparing control versus tick group spectra by period, across planes of nutriton, low discriminant ability was observed (Table 5). Period 4 was the least discriminant (R^2 = 0.06, SECV = 0.48) of this series of comparisons while in period 3 the most difference in fecal spectra was observed (R^2 = 0.36, SECV = 0.40). Unexplainably, validation samples of contol and tick group spectra were correctly identified at similar rates of success (~ 62 versus 68%, 3 versus 4, respectively). Comparing control versus tick groups by period within plane of nutrition yielded results which differed in magnitude but were similar in pattern, i.e. periods 1 and 4 were lower than 2 and 3 (Figure 12.). For the moderate plane of nutrition in all four periods, discrimination between control and tick group spectra was high ($R^2 \geq 0.75$). Correct identification of validation samples was higher ($P < 0.025$, X^2_3) for periods 2 and 3 than for periods 1 and 4. In the low nutrition group, periods 1 and 4 resulted in both low discrimination ($R^2 \leq 0.29$) and validation ability (~ 57%). Period 2, the early tick feeding period, yielded a high degree of discrimination between tick and non-tick treated fecal spectra (R^2 = 0.92), while

Table 4. Discriminant calibration and validation statistics for periods of the tick feeding cycle across both the control and tick-treated groups via near infrared reflectance spectroscopy of feces

Control								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Val. Group	N Val. Smpls.	% Correct
Prd. 1 vs 2	0.91	0.32	1	25	0.84	2	24	0.92
Prd. 1 vs 3	0.92	0.20	1	25	0.92	3	27	0.96
Prd. 1 vs 4	0.94	0.21	1	25	1.00	4	21	1.00
Prd. 2 vs 3	0.85	0.33	2	24	0.96	3	27	0.89
Prd. 2 vs 4	0.89	0.34	2	24	0.96	4	21	0.90
Prd. 3 vs 4	0.82	0.48	3	27	0.89	4	21	0.76
Tick-treated								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Val. Group	N Val. Smpls.	% Correct
Prd. 1 vs 2	0.91	0.30	1	24	0.92	2	23	0.96
Prd. 1 vs 3	0.95	0.24	1	24	1.00	3	27	1.00
Prd. 1 vs 4	0.95	0.24	1	24	1.00	4	21	0.90
Prd. 2 vs 3	0.91	0.34	2	23	0.83	3	27	0.93
Prd. 2 vs 4	0.53	0.41	2	23	0.65	4	21	0.90
Prd. 3 vs 4	0.60	0.41	3	27	0.81	4	21	0.76

a. multiple coefficient of determination

b. standard error of cross validation

period 3, the peak tick feeding period was intermediate ($R^2 \geq 0.56$). Correct identification of samples from tick treated and non-tick treated animals was accomplished at higher ($P < 0.005$, $X^2_{,3}$) success rates in the female tick feeding periods than either pre or post-feeding periods (~73 versus ~ 57%, respectively). Within treatment groups and planes of nutrition, comparing periods, again demonstrated differing abilities to discriminate between groups of fecal NIR spectra (Table 6).

Table 5. Discriminant calibration and validation statistics for the control and tick-treated groups within period of the tick feeding via near infrared reflectance spectroscopy of feces.

Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Val. Group	N Val. Smpls.	% Correct
Period 1	0.24	0.53	Control	25	0.60	Tick-Trt	24	0.46
Period 2	0.23	0.50	Control	24	0.46	Tick-Trt	23	0.39
Period 3	0.36	0.47	Control	27	0.63	Tick-Trt	27	0.63
Period 4	0.06	0.51	Control	21	0.67	Tick-Trt	21	0.71

a. multiple coefficient of determination

b. standard error of cross validation

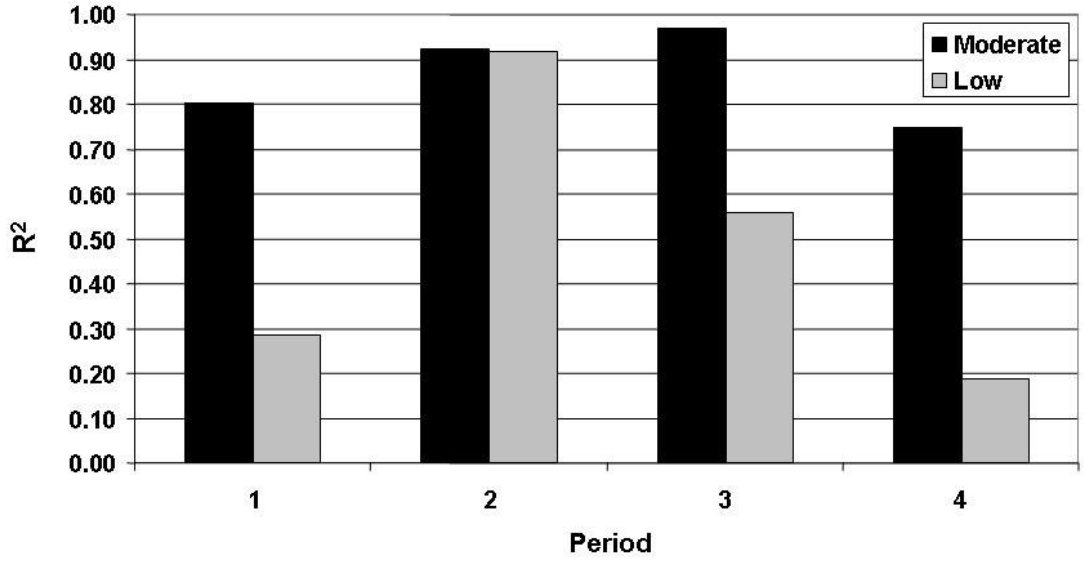

Figure 12. Comparison of coefficient of determination values near for near infrared reflectance spectroscopy of feces discriminant equations: Control versus tick-treatment group by plane of nutrition and period.

Table 6. Discriminant calibration and validation statistics for periods of the tick feeding cycle for both the moderate and low planes of nutrition and within the control and tick-treated groups via near infrared reflectance spectroscopy of feces.

Moderate Control								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Val. Group	N Val. Smpls.	% Correct
Prd. 1 vs 2	0.85	0.32	1	12	1.00	2	12	1.00
Prd. 1 vs 3	0.99	0.18	1	12	1.00	3	14	1.00
Prd. 1 vs 4	0.95	0.20	1	12	1.00	4	10	1.00
Prd. 2 vs 3	0.63	0.42	2	12	0.83	3	14	0.71
Prd. 2 vs 4	0.96	0.43	2	12	0.75	4	10	0.80
Prd. 3 vs 4	0.58	0.37	3	14	0.71	4	10	0.50
Moderate Tick-treated								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Group	Val. Group	% Correct
Prd. 1 vs 2	0.89	0.36	1	12	0.92	2	12	1.00
Prd. 1 vs 3	0.96	0.21	1	12	1.00	3	14	1.00
Prd. 1 vs 4	0.90	0.29	1	12	0.92	4	11	0.82
Prd. 2 vs 3	0.96	0.26	2	12	1.00	3	14	0.93
Prd. 2 vs 4	0.51	0.43	2	12	0.75	4	11	0.82
Prd. 3 vs 4	0.74	0.40	3	14	0.64	4	11	0.55
Low Control								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Val. Group	N Val. Smpls.	% Correct
Prd. 1 vs 2	0.93	0.44	1	13	1.00	2	12	0.83
Prd. 1 vs 3	0.93	0.17	1	13	1.00	3	15	1.00
Prd. 1 vs 4	0.97	0.17	1	13	1.00	4	11	1.00
Prd. 2 vs 3	0.91	0.26	2	12	1.00	3	15	0.87
Prd. 2 vs 4	0.85	0.29	2	12	1.00	4	11	1.00
Prd. 3 vs 4	0.78	0.57	3	15	0.53	4	11	0.73
Low Tick-treated								
Calibration	R^2_a	$SECV_b$	Val. Group	N Val. Smpls.	% Correct	Group	Val. Group	% Correct
Prd. 1 vs 2	0.77	0.35	1	11	0.73	2	11	0.91
Prd. 1 vs 3	0.88	0.21	1	11	1.00	3	13	1.00
Prd. 1 vs 4	0.85	0.25	1	11	1.00	4	10	1.00
Prd. 2 vs 3	0.97	0.30	2	11	0.64	3	13	0.92
Prd. 2 vs 4	0.70	0.42	2	11	0.91	4	10	0.90
Prd. 3 vs 4	0.81	0.49	3	13	0.62	4	10	0.80

$_a$. multiple coefficient of determination

$_b$. standard error of cross validation

In the moderate control groups discriminant extremes are represented by the period 1 versus 4 ($R^2 = 0.99$) and the 3 versus 4 ($R^2 = 0.58$) calibrations. Percent correct validations were 100 and 60, respectively. For the moderate tick-treated groups, extremes in fecal spectra differentiation were periods 1 versus 3 and 2 versus 3 (both $R^2 = 0.96$) with ~ 97% correct validation, and period 2 versus 4 ($R^2 = 0.51$, 78% correct validation). In the low plane control groups period 1 versus 4 ($R^2 = 0.97$, 100%) contrasts with period 3 versus 4 ($R^2 = 0.78$, 60%), while the tick-treated extremes were periods 2 versus 3 ($R^2 = 0.97$, 80%) and periods 2 versus 4 ($R^2 = 0.70$, 90%). Discrimination and validation was also attempted between various random groups of fecal NIR spectra. In this excercise R^2 was always < 0.20 and SECV > 0.45. Percent correct identification of group membership was ~ 50 ± 2 %.

In addition to attempting discrimination between groups of spectra, calibrations for tick drop counts were also attempted with fecal NIR spectra. Calibration equations were developed for daily total female tick drop, daily replete female tick drop, daily cumulative total female tick drop and random numbers between 1 and 100. There were insufficient numbers of daily non-replete female ticks for effective calibration. Leave one out cross validation was employed. Results are presented in Table 7. In the moderate plane of nutrition, the predictive equation for cumulative ticks was excellent with respect to R^2 but the SECV (82.1 cumulative female ticks) represents 27% of the maximum possible value (i.e. 300). Similar results were obtained in the low plane of nutrition group. Calibration R^2 for random numbers (0.53) was similar to that obtained for total

Table 7. Predictive calibration and validation statistics for numbers of feeding female *Amblyoma americanum* within the moderate and low planes of nutrition via near infrared reflectance spectroscopy of feces.

Moderate			
Category	N	R^2_a	$SECV_b$
Replete	54	0.21	38.33
Total	55	0.52	39.37
Cumulative	54	0.93	82.08
Random no's	55	0.53	94.86
Low			
Category	N	R^2_a	$SECV_b$
Replete	51	0.44	26.18
Total	50	0.24	30.98
Cumulative	52	0.96	89.38
Random no's	53	0.11	89.04

$_a$. multiple coefficient of determination

$_b$. standard error of cross validation

ticks (0.52) in the moderate plane of nutrition but the lowest was observed (0.11) in the low nutriton group. By design there were no values comparable to tick counts which could be used in fecal NIRS calibrations with the control group spectra. However, since cumulative numbers were the tick related value most highly correlated with fecal NIR spectra, experimental day was used as a proxy for cumulative tick counts in the control group. Calibration equation results (R^2; SECV) for day (7 to 15) were 0.93; 1.50 and 0.93; 1.89 in the moderate and low plane of nutrition control groups respectively. Corresponding values for random number (7 to 15) calibration were 0.06; 5.85 and 0.20; 6.17, respectively.

Discussion

There were expected differences in fecal NIR spectra between planes of nutrition which would agree with findings by our research group with respect to fecal chemistry attributes in the second paper of this series (Tolleson et al., 2007c). These results contrast sharply with those obtained for random groupings, thus providing a backdrop for discussion of the biological relevance of findings with respect to tick treatment. There were differences in pre-infestation versus infestation fecal spectra within the tick treated groups in both the moderate and low planes of nutrition. These results are in agreement with our previous report (Tolleson et al., 2007a) in which five pen feeding trials, four with cattle and one with horses, infested with different species of ticks and consuming different rations, all yielded "good" or "excellent" fecal NIRS discriminant calibrations for tick versus pre-tick periods. In the same study, developing a calibration with all combinations of four sample sets and predicting group membership (pre-tick versus tick) in the remaining set yielded R^2's > 0.84 but highly variable (0 to 100 %) rates of correct identification of validation samples. Combining all five calibration sets and removing a random 25 % for validation resulted in a high degree of discrimination in the 75% used as a calibration set (R^2 = 0.96, SECV = 0.36) and correct identification of pre-tick (85%) and tick (95%) validation samples. Similar to the current study, calibration for discrimination of random groups was unsuccessful (R^2 < 0.23; SECV > 0.55). The results of this previous study indicate that the possibility exists to apply NIRS

of feces in monitoring tick burdens in livestock. The ability to discriminate between relative tick burdens across tick species, host species, and diets was especially encouraging. This study however lacked non-treated controls.

There were also differences in fecal NIR spectra between tick treated and non treated animals in the current research as evidenced by the high discriminant ability between the two treatment groups during tick feeding periods, regardless of plane of nutrition. This finding corroborates those of another study by our research team (Tolleson et al., 2004) in which a discriminant equation between tick versus non-tick treated fecal spectra (R^2 = 0.71; SECV > 0.42) correctly identified 8 of 10 control and 9 of 10 tick group validation samples. The animals in this previous study were fed the moderate diet utilized in the current research. In both of these studies attempting to evaluate the ability of fecal NIRS as a tool for discerning tick burdens, blood and or blood and liver samples were obtained at important junctures relative to the tick feeding cycle. Thus, as opposed to the multi-sample set, pre versus post study (Tolleson et al., 2007a), the effects of tick stress are confounded with those of confinement, restraint, and venipuncture. In both studies utilizing non-treated controls, there were also varying degrees of discrimination accomplished for non-tick treated animals. For example Figure 13 illustrates differences in princpal component scores of day 7 versus day 14 fecal NIR spectra in the moderate control and tick groups in the current study. Discriminant calibration R^2 values

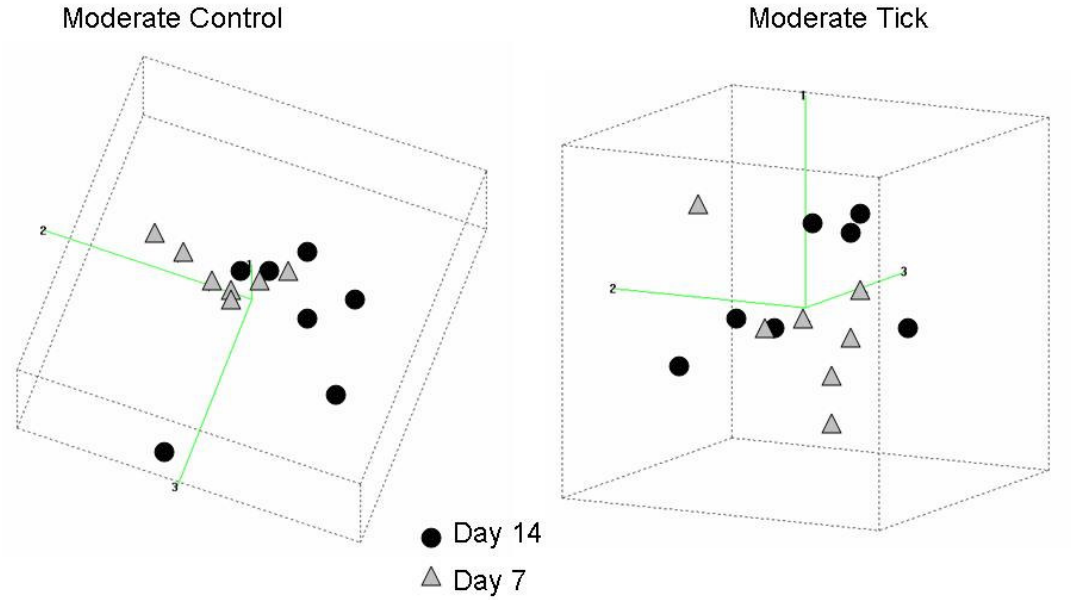

Figure 13. Principal component scores of cattle fecal NIR spectra: Comparison of early and late tick feeding periods within the moderate plane of nutrition.

between days for these two sample sets were 0.72 and 0.43 respectively. There is a greater degree of visible distinction between the day 7 and 14 spectra for the control than the tick group. In both planes of nutrition, especially the low group, disciminant ability was lower during periods not associated with peak tick feeding and blood sampling. Stewart et al., (2007) report an increase in plasma cortisol after jugular catheterization as compared to before. Restraint in a head gate increases cortisol levels as well (Grandin, 1997). In the first paper of this series (Tolleson et al., 2007b) we report that the proportion of tick treated cattle was greater in the highest quartile of cortisol recorded than in the lowest quartile. There were however, representatives of both treatment

groups (tick treated and control) in both quartiles and within a quartile the proportion of each treatment group did not differ dramatically.

There has also been research into the ability of NIRS to identify feces from free-ranging cattle with divergent natural tick burdens (Teel et al., 2004). In this study, cattle grazing in common on native prairie in central Oklahoma were either treated with an anti-acaricide or left untreated. The R^2 and SECV for weekly fecal NIRS discriminant equations indicate that differences in spectra between the treatment groups were lowest on the date of acaricide application (0.24; 0.56), and highest one week later (0.98; 0.36). discriminant ability gradually declined to pre-acaricide values by the fifth week post application (0.17; 0.45. The tick-based NIRS discriminant equation was more accurate (70%) than a random grouping equation (60%, $P < 0.025$). A similar result has been observed by our group using portable NIRS of feces from free-ranging cattle in west-central Texas (P. Teel, Texas A&M, College Station, Texas, personal communication). This research is ongoing.

The ability to predict numbers of cumulative female ticks which had fed to engorgement on these animals was also encouraging. Similar results were obtained with the cumulative tick counts and fecal NIR spectra from our previous study ($R^2 = 0.66$; SECV = 76.67, D. Tolleson, unpublished data). Validation across studies was unsuccessful even between the moderate quality diet groups which was in common between the two trials. Combination of the two different studies moderate diet groups (75% calibration,

25% validation) however, resulted in validation statistics of $r^2 = 0.59$, and SE of prediction = 68.91 (Figure 14). While these equations indicate that ticks are exerting some effect on host metabolism and thus, fecal NIR spectra, the large SE values would render the numerical predictions of little practical value. As with the discriminant calibrations they could be useful for categorizing relative tick burdens.

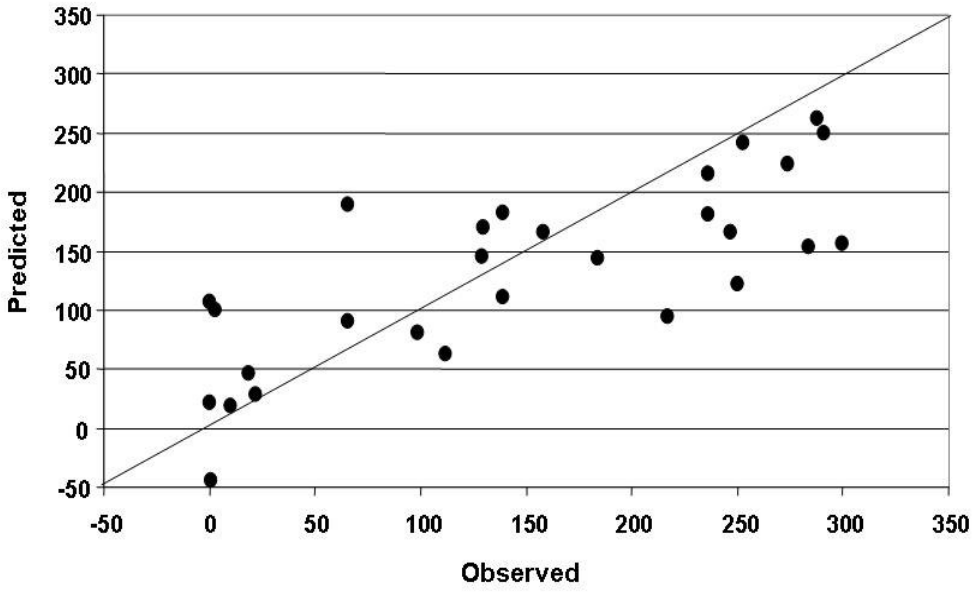

Figure 14. Cumulative daily drop-off of female *Amblyoma americanum*: Observed versus fecal near infrared reflectance spectroscopy predicted values.

This research has neither absolutely confirmed nor rejected the ability of fecal NIRS to discriminate tick burdens in livestock. Taken in context with previous research it woud seem that the ability to utilize NIRS technology as a non-invasive tick monitoring

pocedure certainly seems plausible. For the combined results of this research with regard to animal intake and performance, metabolic indicators, and fecal chemistry, one may also infer that fecal NIRS can potentially be used to monitor other stessors. There were certainly distinguishable differences in fecal NIR spectra between groups not due to tick burdens in this and other studies. What is lacking from all previous research on this topic is a negative control group, one in which there are no ticks applied and in which no invasive sampling methods are employed. Fecal cortisol and NIRS spectra would then be analyzed to detect any differences between these untreated animals and those under stress due to either ticks or invasive sampling. A longer sampling period should also be undertaken. Pen-fed cattle will exhibit cycles of varying intake as normal course (Schwartzkopf-Genswein et al., 2004; Cooper et al., 1999; Caldeira et al., 2007) which could affect fecal constiuents and NIR spectra. The fact that random non-biologically relavent groups or numerical values were predicted lends credence to the hypothysis that stress affects fecal constituents (some of which were measured in this study) through a cascade of metabolic events and that these differences can be detected via NIRS.

CHAPTER V

SUMMARY AND CONCLUSIONS

Monitoring the health and well-being of free-ranging animals is a difficult task. Domestic livestock under extensive management are not easily subjected to the types of intensive, invasive diagnostic techniques often required to obtain such information. Undue stress, prohibitive costs, or difficulty in handling these animals hinder both the implementation and effectiveness of monitoring programs. Nonetheless, society's need for economically sound forage-based animal agriculture demands that the most efficient use be made of annual net primary production from our range and pasturelands. Early detection and alleviation of stress in grazing livestock is one area of rangeland resource management for which there is ample opportunity for improvement.

Stress is difficult to monitor with most conventional diagnostic methods. Frequent blood sampling for instance involves herding or capturing the animals, application of some form of restraint, and ultimately venipuncture. Any of these practices may themselves elicit a stress response thus confounding research results. If they could be imposed less frequently or eliminated, this would greatly improve the quality of stress related research in free-ranging animals. Quantification of blood or fecal entities which could indicate the presence of a stress response *a posteriori* would allow investigators to impose research treatments and allow the subjects to respond to those treatments without additional stress from sampling. The investigator could then evaluate the "evidence" at a later time, in

essence, use “bio-forensics”. To be useful for this purpose a diagnostic compound should: 1) exist in measurable concentrations in blood or feces, 2) in the case of a blood constituent, should posses a sufficient half-life to enable post-stress sampling, and 3) be highly correlated with the stress response. Three studies utilizing a single group of growing beef steers were conducted to ascertain the effects of tick stress on cattle and to evaluate the use of bio-forensic techniques of detection.

Plane of nutrition and tick treatment variously affected animal performance and indicators of metabolism, endocrinology, and immunity as measured in blood. Of these constituents, cortisol and IGF-1 offer possibilities for application in either management of, or research on, animal stress, health, and well-being. Several fecal constituents and characteristics were evaluated to identify, as with blood, any that might provide insight into the physiology of tick stress, or possibly serve as a reliable indictor of such stress. Fecal cortisol was the only candidate to emerge from this effort. The underlying objective of the entire project was to evaluate the ability of fecal NIRS to detect animals with tick burdens. The results were mixed. There are detectable differences in the fecal spectra of animals with and without ticks, or in the same animals before and during a tick infestation. In this research, however, peak tick feeding was confounded with frequent blood sampling. There were also detectable differences in the fecal spectra of non tick-treated animals before and during frequent blood sampling.

This work may have actually posed more questions than it answered. One can not conclude from these results that NIRS of feces is or is not a reliable tool in the management of ticks on free-ranging animals. Ongoing research with tick infested animals on pasture should help to answer this question. One conclusion that can be made is that the effects of "stress" on fecal chemistry can be detected with NIRS. Future studies should be conducted with animals not subjected to intense sampling during the tick feeding cycle. These studies should also employ a longer post-tick sampling period to determine the presence or magnitude of any subsequent effects of tick exposure, i.e. weight gain, immune response or carcass quality.

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